CIRCULATING ISSUE

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B. P.
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BIOCHEMISTRY OF CARCINOGENESIS: A THEORY*

HARRIS BUSCH

Lethal Responses to Stimuli

Cancer is one form of a lethal response to a series of stimuli which have occurred either very recently or sometime previously in the history of the cell. It is well established that various stimuli can be associated with the development of neoplastic disease including *physical factors*, such as ultraviolet light or irradiation, *chemical factors*, such as the carcinogens, *viruses*, i.e. chemicals of an infectious type and other factors such as anoxia. All of these factors are important in producing the lethal response which is characterized as neoplasia. However, it is important to the present concept that cells be considered to respond to stimuli in particular ways depending upon their genetic potentialities, their differentiation and the adjacent functional tissues. It is thus of interest to compare factors causing other types of lethal responses with those causing neoplastic transformation.

An example of another type of lethal response, is *ventricular fibrillation*. Ventricular fibrillation is an abnormality in the function of the myocardium which is characterized by an inability of the heart to pump blood normally. There is a change in the electrical forces in the heart such that the unified flow of electrical currents through the ventricle disintegrates into a large number of foci each one functioning more or less individually. The result of this loss of unity of electrical flow is that instead of the fibers of the myocardium beating together as a single pump, each fiber arhythmically contracts more or less independently. The effect is that the heart simply shimmers. This lack of contractility is incompatible with life because oxygen and nutrition are not transmitted to the various tissues and instead there is a progressive anoxia of all tissues including the heart.

* The substance of this paper was presented at the 1960 Annual Meeting of the Southwest Section of the American Association for Cancer Research. The support for the original studies presented was generously provided by grants from the U. S. Public Health Service, the American Cancer Society, the Jane Coffin Childs Fund, the Anna Fuller Fund. Paper accepted Nov. 10, 1960. (From the Departments of Biochemistry and Pharmacology, Baylor University College of Medicine, Houston, Texas).

This lethal response is essentially a change in electrical characteristics of a given system. It is caused by a variety of factors. Among the *physical factors* are electrical currents, which are probably the most common cause of abnormalities of the ventricle. One way to produce ventricular fibrillation is by a sudden electrical shock to the heart such as is administered in the electric chair. *Chemical factors* also produce this lethal response. One can readily produce ventricular fibrillation in dogs by prolonged anesthesia with cyclopropane or with chloroform followed by a sudden injection of epinephrine intravenously. Another causative factor in ventricular fibrillation is weakening of the myocardium by *viruses* or other infectious agents capable of producing a myocarditis which may be followed by a fibrillation of the heart. Finally, *anoxia* is a most important factor in producing ventricular fibrillation. Small foci of anoxia or large areas of anoxia may be found in patients with myocardial infarction and may be responsible for the production of abnormal foci which suddenly lead to the development of abnormal rhythms, tachycardias and finally ventricular fibrillation.

Thus, lethal responses are by no means limited to neoplastic changes. Tissue responses are conditioned by a variety of factors, which need not be considered as mutations or strange and peculiar aberrations from the norm but are rather as a set of reactions related to the genetic potentialities of the given tissue, the particular stages and degrees of differentiation of the tissue as well as the adjacent functional tissues.

This point of view suggests that the neoplastic process is by no means simply a strange event which occurs occasionally. Like ventricular fibrillation it can occur in any individual and indeed, as the many types of tumors indicate, may occur in any cell. A working hypothesis is that a very definite relationship exists between the genetic character of all cells and their potential character, including neoplastic growth. Many of these potential functions are masked and do not express themselves. On the other hand the potential functions may be stimulated to produce special cell types. This masking or special stimulation is probably one basis for differentiation. It would seem that the ability of cells to manifest themselves as neoplastic cells is a reflection of the release of the genetic potential of these cells from a previously

suppressed state or the potentiation of previously existing potential functions.

Approaches to Protein Metabolism of Neoplastic Cells

Some aspects of the research of this laboratory bear on the hypothesis presented. First of all, it is a very great pleasure to indicate those members of this research group who have been so helpful in developing the research which is indicated below:

S. Simbonis, M.D.
M. Goldberg, Ph.D.
W. L. Nyhan, M.D., Ph.D.
J. R. Davis, M.S., M.D.
E. Fujiwara, M.D., Ph.D.
H. Baltrush, M.S.
W. Starbuck, M.S.
D. C. Firszt, B.S.
E. W. Olle, M.S.
P. V. Nair, Ph.D.
P. Byvoet, M.D., Ph.D.
E. Kohen, M.D.
C. Taylor, B.S.

The line of experimentation to be presented threads its way back to early experiments on labeled albumin (6). The first experiments compared the *uptake of radioactive plasma proteins* by tumors and other tissues (6). The kinetics and the total uptake of labeled plasma proteins were measured by the specific activity of the proteins of the Walker and Jensen tumors compared to the specific activity of the proteins of other tissues. Both the rate and the extent of labeling of tumor proteins markedly exceeded that of other tissues. The specific activity of the proteins of the tumor was three or more times that of the proteins of any other tissue at three hours after the injection of the labeled plasma proteins (6). Later experiments carried out by Dr. S. Simbonis have indicated that serum albumin was the protein taken up to a much greater extent by tumors than by other tissues (8). Control experiments with radioactive amino acids carried out by members of this group and by several other groups did not reveal any significant difference between the uptake of these small molecules by tumors and by other tissues.

These initial studies suggested the need for determination of the *intracellular distribution of proteins and amino acids* labeled with radioactive isotopes. Experiments were carried out *in vitro* by Dr. W. L. Nyhan on the fate of radioactive glutamic acid (14). The efficiency of incorporation of this amino acid into protein of tumors and other tissues was compared. Approximately

11% of the utilized glutamic acid was incorporated into protein of the tumor. In other tissues, less than 1% of the radioactivity of utilized glutamic acid was incorporated into protein. This study suggested that tumors possessed a remarkable efficiency in utilizing amino acids for protein synthesis and pointed up the usefulness of *in vitro* systems for such experiments.

The Nuclear Proteins

The first approach to the determination of the distribution intracellularly of the radioactivity from one of a number of radioactive amino acids involved isolation of a number of cellular fractions (11). Approximately 25% of the radioactivity incorporated into proteins in the liver entered nuclear proteins, while microsomal proteins and proteins of the cytoplasmic sap each contained about ⅓ of the radioactivity incorporated into proteins. In the Walker tumor, on the other hand, approximately 55% of the total radioactivity incorporated into proteins was incorporated into nuclear proteins. The proteins of the cytoplasmic sap again contained approximately one-third of the total radioactivity incorporated into proteins. However, the microsomal proteins of the tumor contained only 9% of the total radioactivity.

Chromosomal organization.—These data suggested that the tumor was specializing in the biosynthesis of nuclear proteins and led to experiments designed to determine where in the cell the nuclear proteins were being formed and what types of substances were biosynthesized. Sirlin and Knight (15) have recently provided evidence for protein synthesis directly on the chromosomes in small bands which seem to appear on the chromosome in much the way that the various striations appear to cross the skeletal muscle. An inquiry into the nature of the chromosome and the type of proteins which might be biosynthetized in the nucleus led to a literature survey which was presented some time ago (3). A variety of concepts exist at present with regard to the structure of the chromosome (2). In one, the chromosome is considered to be a three-layered system with a central core of protein and a middle layer of ribosephosphates and a surface layer of purine and pyrimidine bases. This concept is no longer held because the bases of nucleic acids are not believed to be on the surface of the molecule but rather are bonded together within the molecule. Another concept of the structure of the chro-

mosome is that it consists of alternating bands of protein and nucleic acid, in which proteins may serve to cement together the nucleic acid bases. This concept would suggest a role for the proteins in holding the structure together, but would not assign to the protein any particularly important role in genetic transmission. The most recent concept of Zubay and Doty (17) suggests that there is a two-layered system consisting of a central core of purines and pyrimidines, and a middle layer consisting of ribose-phosphates. To this surface are attached the nuclear proteins which then might have an important genetic function in the sense that they might there serve as suppressors or stimulators for specific genetic loci. This concept would assign considerably greater importance to the nuclear proteins than had hitherto been suggested. The view is supported by the fact that the molecular weight of nucleic acids is generally believed to be approximately 8×10^6 while the molecular weight of the nuclear proteins is approximately $30 - 50 \times 10^3$. The nucleic acid would then be a long strand to which proteins are attached.

Types of nuclear proteins.—It seemed logical to ask what types of nuclear proteins are there (3)? Sometime ago it was believed there were four or five types which could be separated from one another by differences in solubility. These different types included the *histones* which were extractable from isolated nuclei with acid and *acid-insoluble* proteins which comprised residual proteins, globulins, lipoproteins and NaCl-soluble nuclear proteins. These substances were not particularly well characterized and although a large number of important measurements on their characteristics have been made, adequate evidence for their purity had not been obtained.

Biosynthesis of Nuclear Proteins

Initial experiments required determination of the intracellular distribution of radioactivity *in vivo* and *in vitro* following the administration of radioactive amino acids to tumor-bearing animals or following the incubation of tissue slices with radioactive amino acids. In general, it was determined that the specific activity of the cytoplasmic proteins was uniformly very high in nontumor tissues compared to the specific activity of the nuclear proteins. This result was obtained *in vitro* in the pancreas, spleen, liver, regenerating liver, diaphragm and kidney in studies carried

out by Mr. Wesley Starbuck (16). However, the specific activity of the nuclear proteins, particularly the histones, was equal to or greater than the specific activity of the proteins of the cytoplasmic fractions in each of the tumors studied including the Walker tumor, the Jensen tumor, and the Ehrlich ascites tumor.

Similar studies were carried out *in vivo*. Following the injection of radioactive lysine into tumor-bearing animals, it was found that the specific activity of the histones was higher in the Walker tumor than the specific activity of other cellular fractions (4). In liver, kidney, pancreas, spleen, intestine and other tissues, the specific activity of the cytoplasmic proteins was uniformly much greater than that of the nuclear proteins. To determine whether this result was a general one for amino acids, further studies were made with seventeen radioactive amino acids (5). It was found the specific activity of the histones was approximately equal to that of the most radioactive cytoplasmic protein in the Walker tumor, while in each of the other tissues studied, the specific activity of the histones was approximately half that of the most radioactive cytoplasmic fraction. In comparison with the specific activity of the proteins of the whole homogenate, the specific activity of the histones was 1.2 times that of the proteins of the whole homogenate in the Walker tumor. In the other tissues studied, the specific activity of the histones was 0.7 or less than that of the proteins of the whole homogenate. These data lead to the obvious conclusion (4) that the amino acid pool in nontumor tissues was being used for synthesis of the special proteins which are products of those tissues. For example, the liver is forming albumin and other plasma proteins. The kidney is forming enzymes for tubular resorption; the pancreas is forming zymogen granules and digestive enzymes. On the other hand, the Walker tumor would appear to be actively synthesizing nuclear proteins.

To test further the factors which made nuclear protein synthesis a very active function of the tumor tissues, experiments on the nature of these proteins were initiated. To do this, a variety of techniques were required, beginning with chromatographic methods (12). Manifold techniques were employed with as many as eight columns being run simultaneously (11, 12). In addition, manifold fraction collectors were used so that the process of collection of samples could be made almost entirely automatic.

Furthermore, the methods for isolation of nuclei were now adjusted to provide samples from tumors for the first time in which the nuclei were virtually completely free from cytoplasmic granules (9).

Abnormal Nuclear Protein Synthesis in Tumors

The fundamental feature of the chromatographic method was elution of the proteins from carboxymethylcellulose columns with the aid of increasing concentrations of formic acid (7). The chromatographic patterns (Fig. 1) obtained plotted fraction number on the abscissa and either radioactivity or optical density on the ordinate (11). In the patterns for the nontumor tissues one of the fractions uniformly found in the tumors, i.e. radioactive peak 2 (RP2-L), was missing. In each tissue 5 to 10 protein peaks were found. When these patterns were compared with the distribution of radioactivity for the individual tissues, specific patterns were found for intestine, liver, thymus, pancreas, spleen, and other tissues. In regenerating tissues, such as regenerating liver, or the growing nontumor tissue, such as embryonic tissue, 5 to 8 protein peaks were found. In regenerating liver the only radioactive peak found was peak 3 (13). In embryonic tissues, only radioactive peaks 1 and 3 were found. In each of the tumors, however, including the Flexner-Jobling carcinoma, the Jensen sarcoma, a human malignant melanoma, the Walker 256 carcinosarcoma, Sarcoma 180, and the Ehrlich ascites tumor there was a radioactive peak (RP2-L) which has not been found in non-

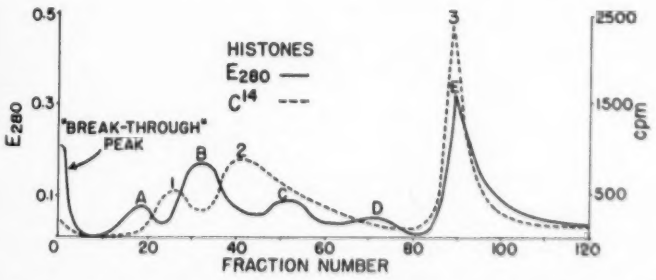

FIG. 1. Chromatographic analysis of the acid-soluble nuclear proteins with radioactivity and absorption at 280 mu as the parameters. The radioactive peaks are indicated in arabic numerals and the protein peaks are indicated in block letters (11).

tumor tissues (13). Inasmuch as labeled lysine was the precursor and this peak was the second radioactive peak found in samples of Walker tumor, it has been designated as RP2-L, i.e. radioactive peak 2 obtained with L-lysine-C^{14} as the tracer.

Recent efforts have been made to purify this protein by rechromatography. Such procedures were initially successful in providing fractionation for these preparations and isolation of these proteins in reasonably high states of purity has been achieved. With these preparations, Dr. Davis has determined the diffusion constant and sedimentation constant for the purest sample obtained to be 4.58 and 1.79 respectively. The molecular weight determined by physical methods was 34,000 and is in agreement with the molecular weight calculated from data on amino acid analysis. The total number of amino acid residues is approximately 300 in RP2-L and the purity of the most purified preparation obtained is approximately 96%.

In a further effort to determine the intranuclear location of RP2-L, Dr. Byvoet has carried forward experiments on the precipitation of these proteins from various chemically isolated segments of the nuclei of the Walker tumor. The method employed was designed for isolation of chromosomal proteins. Initially, the nuclei were treated with 0.15 *M* sodium chloride. The extract contains the bulk of the proteins of the nuclear sap but low concentrations of the DNA bound proteins, i.e. the chromosomal proteins or histones. The proteins of the extract and the precipitates were either dialyzed against or extracted with hydochloric acid. The acid-soluble proteins were then chromatographed on carboxymethylcellulose columns. A peak which appears to be RP2-L was found in the fraction which was isolated from the chromosomal or DNA bound protein, but was not present in the extract of the nuclear sap.

A Theory for Carcinogenesis

It is possible to interpret the data presented above in terms of a working theory of carcinogenesis. The inherited potentiality for cells to become cancer cells must be related to their genetic potentialities and must be conditioned by their function. For example, fully differentiated brain cells may not ever have the opportunity to become cancer cells. However, many cells must possess the capacity to become cancer cells and the neoplastic

state is expressed in most tissues and organs following a variety of stimuli which can produce the neoplastic change. Multiple neoplasms in a given patient are not uncommon and many tissues in the same animal or patient may develop the neoplastic state. When one injects acetylaminofluorene into animals as many as five to eight types of tumors may develop and hence the potentiality for neoplastic development must be genetically inherited and widely distributed in somatic cells. Such findings indicate that there are genetic loci already present in virtually every cell which become expressed as the neoplastic state under the right circumstances.

The types of symbols used for illustrating the various components of the process are indicated in the accompanying chart (Fig. 2). The fundamental role of the carcinogen in the *process*

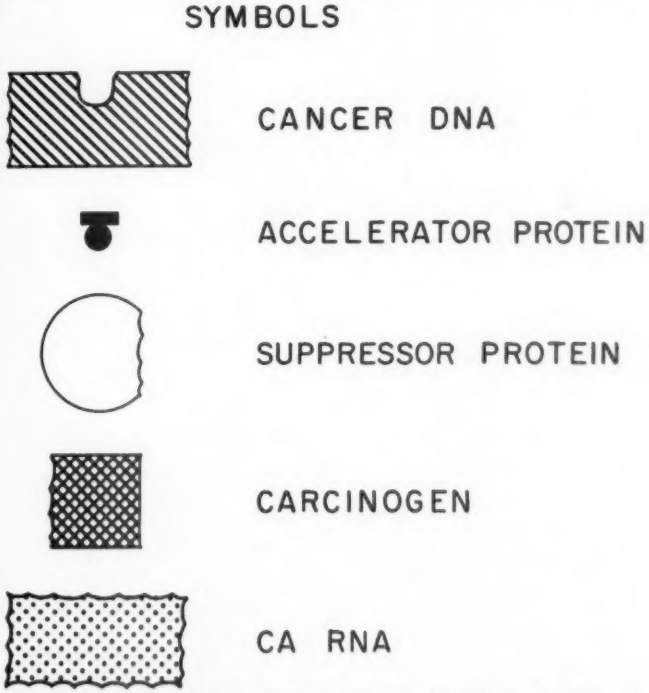

FIG. 2. Symbols used in the following figures to indicate the factors involved in carcinogenesis.

of initiation of neoplastic disease (Fig. 3) is visualized as one of three possible types of effects. Cancer loci in DNA or the specific cancer DNA capable of producing the neoplastic process is released from a suppressed state by a variety of combinations of carcinogen with either the DNA or the suppressor (Fig. 3). One possibility (I) indicated is an attachment of the carcinogen to inactive cancer DNA. This inactive cancer DNA is still combined with the suppressor and hence it is still inactive but it is altered by the presence of the carcinogen. A second possibility (II) is the combination of the carcinogen with both the suppressor and the cancer DNA. In these two types of reactions there is some weakening in the binding of the DNA to the suppressor. In the third type of reaction, active cancer DNA would result, since the sup-

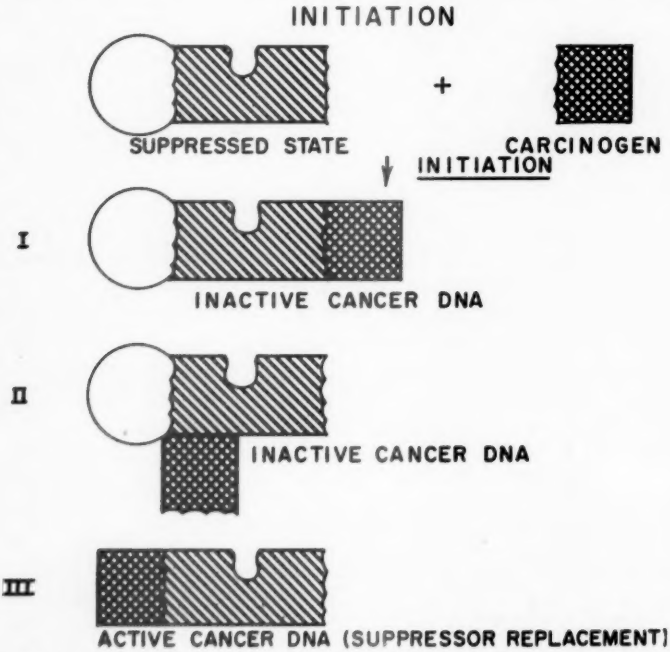

FIG. 3. Stage of initiation of carcinogenesis. Three different types of attack on DNA by the carcinogen are indicated. I is a combination of carcinogen with DNA, which remains combined with suppressor. II is a ternary combination of carcinogen with DNA and suppressor. III is a replacement of the suppressor by the carcinogen.

pressor has been displaced from the surface of the DNA. Either the suppressor is pushed off the molecule by the carcinogen or it is combined with the carcinogen temporarily but long enough to remove it from the surface of the DNA. This last process would obviate the need for the *stage of promotion* generally considered to be the second stage in the production of neoplastic disease because the DNA in the third type of reaction is already active.

The *stage of promotion* in the neoplastic process is related to release of suppressor by a combination of the suppressor and the carcinogen in such a way that the active cancer DNA is released (Fig. 4). Such DNA might either be attached to carcinogen or might be free of both carcinogen and suppressor, i.e., attached to neither. In the type of combination indicated in *suppressor release* the promoting agent would produce a change such that there would be an alteration of bond strength between suppressor and the carcinogen-linked DNA with a resultant release of suppressor. In the second type of complex, combination has occurred of the suppressor with the carcinogen; the promoting factor would enhance the breaking of the bond between carcinogen suppressor complex and DNA. Then there is release of active cancer DNA.

This concept suggests that active cancer DNA would initially

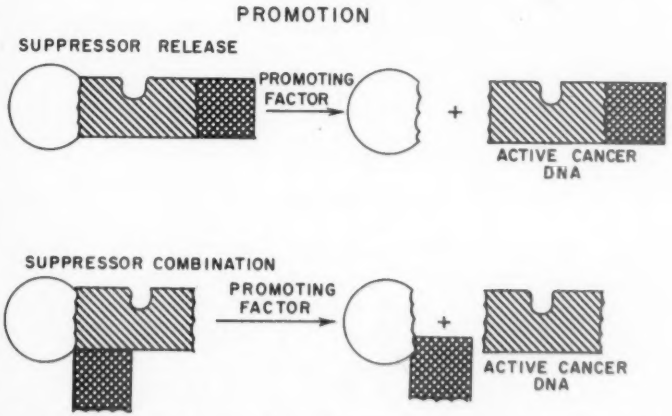

FIG. 4. Stage of promotion in carcinogenesis. The promoting factor may produce release of the suppressor from the complex of suppressor-cancer DNA-carcinogen, or the promoting factor may cause a release of the suppressor-carcinogen complex from the ternary complex of suppressor-cancer DNA-carcinogen.

be a single genetic locus in the cancer cell. The locus would be very small and the reaction rates involved in the next processes would be very, very slow because of the small amount of active cancer DNA available. In the *stage of acceleration* (Fig. 5) the cancer DNA which was slowly formed over a long period of time, is now released from the inhibition and slowly forms cancer RNA. It is generally believed that one of the biochemical roles of DNA is the formation of RNA which carries the genetic stamp into the cytoplasm. Normally RNA is believed to be the template for specific protein synthesis. The cancer RNA would presumably differ from other cellular ribonucleic acid.

The function of the cancer RNA would be to induce the formation very slowly of cancer protein which is shown symbolically (Fig. 5) to be able to fit as a cofactor in DNA. This protein would activate and accelerate the process of formation of cancer RNA (Fig. 5) which would in turn further accelerate the formation of cancer protein.

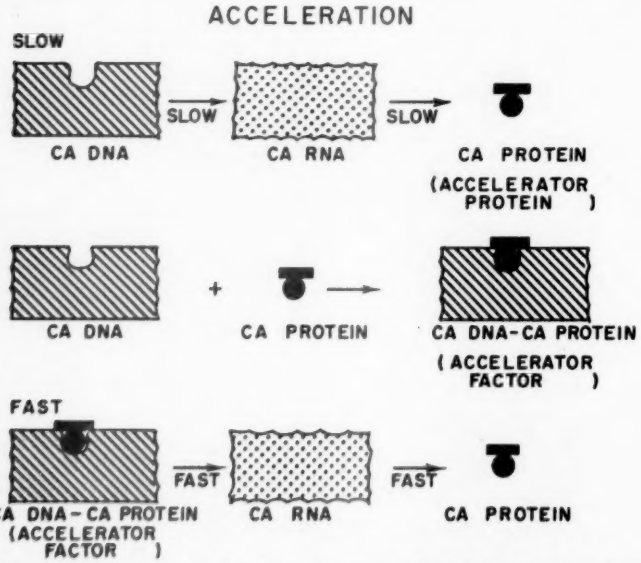

FIG. 5. Stage of acceleration in carcinogenesis. Initial slow reactions leading to the formation of cancer RNA and the accelerator protein. The accelerator protein functions as a cofactor for the rapid formation of cancer RNA which in turn forms more of the accelerator protein.

The rate-limiting factor in this cyclical process would be the active cancer DNA. It may be envisioned that in the cell there is much cancer DNA which is normally suppressed and that the formation of cancer protein proceeds at a negligible rate, if at all, in view of the fact that cancer RNA is not formed. Once the process of rapid formation of cancer protein has occurred there would be a very rapid conversion of all the DNA in the cancer cell to cancer DNA regardless of whether or not suppressor was still attached. The increased availability of cancer protein would cause most or all available cancer loci in the DNA in the cell to become converted to active loci. Competitively, this process would supersede all the preexisting processes for biosynthesis of normal cytoplasmic protein elements and protein templates such as the ribosomes.

These changes would be reflected in altered mitosis, increase in the amount of abnormal DNA, increase in the formation of abnormally large amounts of RNA, and the formation of abnormal nuclear protein. The evidence for these three changes then, would be then related to the cardinal findings in neoplastic disease. The first is the histological evidence for the nature of neoplastic disease, i.e., an increase in size, shape and staining characteristics of the nucleus. Secondly, Casperson and Santesson (10) have indicated that one of the most constant features of neoplastic disease is the increase of the size of the nucleoli. Finally the data presented in the review of studies of this laboratory have indicated the presence of abnormal proteins in neoplastic cells, which may be the accelerator proteins (12, 13).

Analogies to Release of Suppressor Systems

The concept of progressive acceleration of biological process through changes in the suppressor mechanisms is by no means new (1). The suppressor mechanisms for prevention of *pancreatic destruction* are already well known and are related to inhibitors for tryptic digestion of the gland. Trypsin is responsible for the development of acute pancreatitis. The release of the tryptic activity may occur by virtue of the decreased concentration of trypsin inhibitor in the cell (1). As a result of this decrease in suppressor concentration, the autocatalytic activities involved in release of trypsin from trypsinogen rapidly increase the concen-

tration of free trypsin to the point where progressive digestion of the gland occurs.

The *blood clotting mechanism* has a variety of cofactors involved in the formation of active enzymes converting fibrinogen to fibrin and suppressor mechanisms which inhibit the activity of thrombin and inhibit its formation. Normally, these reactions proceeding under the control of many systems. Under special circumstances, fibrinogen is converted to fibrin at a rate which is very rapid. Of the many factors involved in this delicately balanced system, there are calcium, thromboplastin, proconvertin, accelerin, a variety of special globulins and many suppressor substances.

In the cancer process, the beginnings are very slow and the availability of suppressor substances are such that the reaction even once begun is relatively slow. Once, however, the accelerators are released and suppressors are lost, the pace of cell division increases rapidly.

Any theoretical approach to a problem is of value only in terms of the experiments it suggests. This theoretical basis for carcinogenesis suggests that through inhibition of the formation of the cancer protein (the accelerator protein) an inhibition of formation of the new cancer DNA will result and with this a suppression of the neoplastic process. For example, if peptides or large molecules such as albumin derivatives can enter the cancer cell and can carry with them the proper configurations to suppress the formation of accelerator proteins from cancer RNA, a very useful approach to therapy might be developed. On the other hand, if proper peptides could substitute for the cancer protein on the affinity points or reactive centers of the cancer DNA the proper fit might suppress the activity of cancer DNA. Indeed, the presence of lethal groups could conceivably destroy the cancer DNA. Only the proper experiments can determine whether such an approach will be effective. In any event, all of these considerations point to the need for progressively increased research in the area of the functions of nuclear proteins, their formation, their metabolic activities and finally their roles as cofactors or informational agents within the cell.

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THE FIBRINOLYTIC SYSTEM: A REVIEW OF ITS THERAPEUTIC SIGNIFICANCE AND CONTROL, WITH PARTICULAR EMPHASIS ON ITS INHIBITION BY E-AMINOCAPROIC ACID[a]

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Physiologic Aspects

In the last three decades, important advances have been made in our understanding of the mechanisms by which fibrinolytic activity develops in the plasma of a variety of mammalian species (9, 34, 65). At the present time, particular emphasis is being placed upon the operation of this system in man with the goal of controlling the deposition of fibrin in hypercoagulable states as well as bringing about the dissolution of newly formed intravascular fibrin clots (20, 64). The fibrinolytic system as currently conceived consists of an enzymatically inactive precursor, profibrinolysin or plasminogen, which can be converted into an active proteolytic enzyme called fibrinolysin or plasmin.

Activation of the proenzyme can be effected by a variety of agents. Some of these are presumably non-enzymatic and involve the treatment of profibrinolysin with micromolecular agents such as chloroform (39). Activation under more physiological conditions is achieved by various macromolecular activators such as exotoxins produced by certain bacteria, e.g., staphylokinase (37) and streptokinase (17), and by agents produced within the mammalian organism of which various tissue activators (11) and urokinase (10, 14, 69) comprise the most familiar examples. The presence of activators in plasma also has been claimed (48). More recently it has been reported that in the presence of 50% glycerol, profibrinolysin, over a rather prolonged period of time, is converted to fibrinolysin by a mechanism which is purported to be autocatalytic (3).

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The precise physiologic role played by the fibrinolytic system in health is not known. It has been suggested as a participant in immunological reactions; e.g. C'1 of complement has been reported to be activated by fibrinolysin (36). However, its primary function would appear to be the prevention of intravascular accumulation of fibrin (9). Fibrin is believed by some (8, 21, 56) to be formed in small amounts within the vasculature under physiologic conditions.

Clinical Considerations

Even without a complete understanding of the physiologic role of fibrinolysin, the controlled development of sufficient fibrinolytic activity *in vivo* to remove detrimental intravascular clots has long been deemed desirable and feasible. However, in a pharmaco-dynamic approach to the dissolution of intravascular coagula, two important factors must be considered: (1) the difficulty of obtaining appropriate activators and/or exogenous fibrinolysin; and (2) the need to overcome potent physiologic inhibitors of fibrinolysin present in plasma. Among the inhibitors, the most important from a quantitative viewpoint is antifibrinolysin. This agent, which appears in abundance in the plasma albumin fraction (40), materially reduces the effectiveness of fibrinolysin whether it is formed intravascularly or administered preformed. Two other inhibitors of fibrinolysin (or of its activation) have also been reported to be present in the alpha-1 and alpha-2 globulin fractions of plasma (31, 52, 61).

Within the last three years, potent preparations of activators, particularly highly purified streptokinase (20, 24, 70, 73), as well as some preparations of human fibrinolysin itself (6, 7, 12, 13, 19, 20, 22, 24, 28, 30, 33, 45, 46, 58–60, 70, 72, 73, 76, 77) have been subjected to clinical trial. The results of these tests, while in the main encouraging, have presented new problems. Foremost among these has been the realization that adequate methods are not available which will permit the clinician to follow on a moment-to-moment basis the fibrinolytic state of his patient. As a consequence, excessive fibrinolytic activity has occasionally developed during the treatment of patients with streptokinase and/or fibrinolysin.

Even in the absence of exogenous activators or fibrinolysin, excessive fibrinolytic activity may develop in certain pathologic

states; in such circumstances the causative agent is usually unknown although fibrinolytic states following lung and prostatic surgery (38, 63, 71) are believed to result from one or more tissue activators entering the circulation in considerable quantity.

While apparently not serious in itself, such "runaway" fibrinolytic activity (occurring either spontaneously or as the result of enzyme therapy) is frequently accompanied by the development of circulating anticoagulants (24, 75) which are believed to be the result of products of partial digestion of fibrin (24, 49) and possibly other plasma proteins as well. Such partially degraded proteins may interfere not only with thromboplastin generation and thrombin formation but also with the polymerization of the fibrin monomer (23). In addition to substances which interfere with the coagulation mechanism, actual defects in the coagulation mechanism may occur as a result of the destruction of key components such as factor V (6, 13, 20). The simultaneous presence of the fibrinolytic state and impaired coagulation may result in massive hemorrhage and constitutes a potential major hazard in the therapeutic use of the preparations of exogenous activators and fibrinolysin which are now available for clinical use. Although we, as well as others (25), have been unable to verify *in vitro* the potency claims for certain lots of human fibrinolysin preparations marketed by two different pharmaceutical houses, there is clinical evidence (6, 13, 19, 22, 24, 28, 30, 33, 58, 59, 70, 76, 77) that the use of these preparations, as well as such exogenous activators as streptokinase, has occasionally been accompanied by the development of an excessive fibrinolytic state *in vivo*. If these agents are to be used with safety, even by those with extensive facilities for assaying the fibrinolytic status of the patient, means must be at hand which will permit the clinician to decrease *in vivo* fibrinolytic activity rapidly and effectively. Furthermore, in the treatment of spontaneous fibrinolytic states, the use of an inhibitor of fibrinolysin and/or activators may be therapeutically desirable.

Until recently the only agent known to inhibit fibrinolysin effectively *in vivo* was antifibrinolysin which has been supplied for clinical use in the form of a low salt, human serum albumin solution (55, 63) or pseudoglobulin fraction (68). Highly purified, pyrogen-free preparations of antifibrinolysin are difficult to obtain. The only source consists of human plasma with the ever-

present possibility that the antifibrinolysin preparation will contain the virus of serum hepatitis as well as occult antigenic material. Like most protein molecules, antifibrinolysin is ineffective when taken orally and individual idiosyncrasies to it cannot be readily predicted. An effective *in vitro* inhibitor of fibrinolysin (18) occurs among the proteins of soy bean and has been highly purified (35). However, a number of considerations, particularly its *in vivo* toxicity, have to date precluded its use.

E-Aminocaproic Acid Inhibition of Fibrinolytic Activity

Since the issuance in 1957 of a British use patent (43) to a Japanese chemical company, there has been considerable attention directed to the micromolecule, epsilon-aminocaproic acid (E-ACA), as an agent which appeared feasible for the *in vivo* inhibition of fibrinolytic activity. In the British patent, it was pointed out that among the compounds tested, E-ACA possessed singular characteristics as an *in vitro* inhibitor of the fibrinolytic system. The patentees administered 10 to 20 grams of this compound daily to humans and observed no toxic symptoms. They claimed E-ACA was beneficial in the treatment of morning sickness, dysmenorrhea, threatened abortion, toxemia of pregnancy, and various allergic disorders. However, they did not establish it as an *in vivo* inhibitor of the fibrinolytic system. Similar claims were made by the same workers in the application filed in November, 1953, with the United States Patent Office which issued a patent in June, 1960, for the use of E-ACA as a method of treating diseases associated with plasmin activity (44).

Reports of the effectiveness of other micromolecules related to E-ACA as inhibitors of fibrinolysin have been scattered. In 1952, Ginsburg *et al.* (27) reported that polypeptides of lysine and similar compounds were effective *in vitro* inhibitors of fibrinolysin. Müllertz, in 1954, observed that lysine and arginine as well as other amino acids could inhibit fibrinolysin (47); however, the antifibrinolytic potency was weak and the report was relatively unnoticed. In 1955, Troll and Sherry (74) observed that esters of lysine, particularly lysine ethyl ester, inhibited the conversion of profibrinolysin to fibrinolysin in the presence of streptokinase. Methylamine has been reported by Norman (51) to inhibit the caseinolytic activity of human plasmin.

The work of the Japanese investigators reported in the British

patent of 1957 (43) stimulated considerable interest. In 1959, several papers appeared in which E-ACA was studied *in vitro* as an inhibitor not only of fibrinolysin but also of its generation from profibrinolysin in the presence of various activators. In the first of these, Ablondi *et al.* (2) confirmed the report that E-ACA could inhibit fibrinolytic activity and pointed out that it was a potent inhibitor of the conversion of profibrinolysin to fibrinolysin by activators. His work also indicated fibrinolysin was somewhat more stable in the presence of E-ACA than in its absence. Alkjaersig *et al.* (4, 5) used E-ACA as a tool in *in vitro* studies designed to further elucidate the mechanisms of activation of fibrinolysin. They reported that E-ACA was highly effective as an inhibitor of enzymatic activators of profibrinolysin and inhibited fibrinolysin only in high concentrations. In low concentrations, E-ACA actually appeared to enhance the activity of fibrinolysin. The anti-activator action was further emphasized in a review by Sherry, *et al.* which appeared in April, 1959 (65). Sjoerdsma and Nilsson, in 1960, studied a series of aliphatic amino compounds as inhibitors of profibrinolysin activation and concluded that the presence of a terminal amino group was required for activity. They also reported that E-ACA did not inhibit the action of fibrinolysin on heated fibrin plates.

In December, 1959, the work of the Japanese investigators was presented in detail in a series of papers (1, 16, 29, 42, 53, 54, 62, 78, 79) which included a review by Okamoto (53) of the physiologic and clinical significance of plasmin (i.e. fibrinolysin) and its inhibitors, particularly E-ACA. Abe and Sato (1) reported that E-ACA stoichiometrically inhibited fibrinolysin and tissue activator but had no effect on trypsin.

Fukutake *et al.* (26) in May, 1960, published the results of *in vitro* studies which led them to postulate a two-step reaction scheme for the action of fibrinolysin and to conclude that the antifibrinolytic properties of E-ACA are exerted primarily in the first phase of fibrinolysis which involves the solubilization of fibrin. They found that E-ACA had little effect on the action of fibrinolysin in the second phase of the proposed mechanism which involved the further digestion of the soluble products, fibrinolysopeptide and metafibrin.

As a consequence of recent experiments, detailed in an accompanying paper (15), Celander *et al.* have concluded that (1)

E-ACA definitely inhibits fibrinolysin; (2) the sensitivity of the fibrinolysin to E-ACA inhibition varies with the species of origin; (3) the degree of sensitivity of activators to E-ACA is not uniform, both urokinase and staphylokinase being markedly less sensitive than the streptokinase-elicited human or canine activators; and (4) the amounts of E-ACA required to inhibit activators effectively are sufficient to bring about marked inhibition of fibrinolysin itself. This latter effect has been unjustifiably deemphasized in the recent literature.

Although E-ACA is not yet in common use clinically, its efficacy has been tested in a wide variety of experimental and clinical situations (1, 16, 29, 32, 41–44, 50, 54, 62, 66, 78, 79). Okamoto *et al.* (54) compared the effectiveness of E-ACA in suppressing bleeding from cut surfaces in (1) dogs which had been made intensely fibrinolytic through the intravenous injection of human serum (5 ml./kg.) and streptokinase (7,000–10,000 units/kg.) and (2) dogs which were subjected to anoxia following heparinization (4 mg./kg.). E-ACA was effective in stopping the bleeding due to increased circulating fibrinolysin but failed to stop oozing from cut surfaces in the heparinized, anoxic dogs. These latter animals showed no increase in circulating fibrinolysin.

Control of dysfunctional uterine bleeding has been reported (62) with the use of 1 to 5 daily injections of 5% E-ACA (20 ml.). Bleeding from mucosal surfaces (gums, nasal passages) and petechial bleeding associated with aplastic anemia and with leukemia disappeared following the administration of E-ACA either alone or in combination with prednisolone (42). In these cases and in a series reported by Abe and Sato (1), the hermorrhagic tendencies were associated with high plasma fibrinolysin titers. It is of interest that Abe and Sato (1) also found E-ACA to be effective occasionally in controlling hemorrhage in patients with low fibrinolytic indices. Sherry *et al.* (66) found that E-ACA was capable of preventing the induction *in vivo* of fibrinolytic activity by Pyrexal, nicotinic acid, or streptokinase and could abolish fibrinolytic activity already induced by these agents.

An increase in urinary fibrinolytic titers following exposure to small doses of gamma-radiation was noted by Chien *et al.* (16) and by Yamashita and co-workers (78). Both groups found that the urinary fibrinolytic activity was inhibited *in vitro* by E-ACA

and the latter group found that if 20 ml. of 5% E-ACA was injected prior to exposure, the increase in urinary fibrinolytic activity was not observed. However, it should be pointed out that E-ACA is excreted, apparently unchanged, at a fairly high rate via the kidneys (50). Since the urine samples were not dialyzed before assay, their E-ACA content could presumably have prevented the detection of any increase. Normally human urine contains only traces of fibrinolysin. The claim made by Yamashita *et al.* (78) that both fibrinolysin and activator (urokinase) are present in human urine following exposure of the subjects to minute doses of radiation is of considerable interest.

The effective employment of E-ACA against urokinase activity was reported by McNichol *et al.* (41) at the meetings of the Federation of American Societies for Experimental Biology in April, 1960. These workers administered parenteral E-ACA to patients who had undergone transuretheral prostatic resection and found that the prolonged postoperative bleeding and clot sloughing which frequently accompanies such surgery, was materially reduced in the 5 patients studied. As a consequence, the patients suffered much less discomfort and their hospital stay was shortened. Examination of the undialyzed urine of these patients revealed a complete absence of urokinase activity. However, when the urine was freed of E-ACA by dialysis, urokinase activity could be demonstrated. They concluded that E-ACA interfered neither with the production nor the excretion of urokinase but only with its action.

At the same meetings, Johnson *et al.* (32) reported on the use of E-ACA in four patients exhibiting chronic excessive fibrinolytic activity with "hypofibrinogenemia and/or gross hemorrhage." The plasma of all four patients showed increased concentrations of activator and decreased concentrations of inhibitors to activators. Following the infusion of these patients with amounts of E-ACA calculated to inhibit circulating activator, whole blood clot lysis and hemorrhage disappeared. Examination of plasma revealed a return to normal of plasma activator and inhibitor. These authors concluded that the abnormal state of their patients was due to an insufficient amount of activator inhibitor.

Nilsson *et al.* (50) in June, 1960, reported the successful use of orally administered E-ACA in the treatment of two patients in which hemorrhage was associated with excessive fibrinolytic

activity. In a third patient with an elevated level of plasma activator they were able to demonstrate anti-activator characteristics of E-ACA. These workers used 16 to 36 grams E-ACA per day given orally in four to six divided doses. E-ACA was excreted rapidly by the kidneys (from 65 to 97% of a given 12-hour dose being excreted in 12 hours). In one patient in this series, with uremia and presumed renal dysfunction, the inhibitory effect of E-ACA persisted for a considerably longer period than in patients with normal renal function. The authors emphasize the need for caution in administering this compound to any patient with suspected renal impairment.

From the foregoing, it is apparent that E-ACA has been of value in the study of biochemical and physiologic aspects of the fibrinolytic system and appears to hold considerable promise as an effective therapeutic agent for the control of hemorrhagic manifestations associated with the presence of excessive fibrinolytic activity (either therapeutically induced or spontaneous). Attention of investigators has yet to be focused on the information which may be derived, through the judicious *in vivo* use of E-ACA in animals and man, concerning the true physiologic role of the fibrinolytic enzyme system. Until this information is obtained and used to evaluate the effects of even partially inhibiting the fibrinolytic system, we believe that the excessive use of agents, such as E-ACA, which inhibit fibrinolysin itself and its activation, should be approached with caution.

Summary

Activation and activity of the fibrinolytic enzyme system, both *in vivo* and *in vitro*, are reviewed briefly. Attention is directed to the current, widespread use, therapeutically, of potent activator preparations and fibrinolysin and to the clinical problems which have been encountered. Epsilon-aminocaproic acid (E-ACA), a micromolecular inhibitor of the fibrinolytic enzyme system, has been employed to study the biochemical and physiologic aspects of this system. Its usefulness in the control of hemorrhagic manifestations associated with the presence of excessive fibrinolytic activity appears to be well documented. However, the authors urge caution in the use of this compound in conditions in which excessive fibrinolytic activity is not present or, if present, cannot

be established definitely as a causative agent, and in cases involving impaired kidney function.

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PROCEEDINGS OF THE TEXAS BRANCH OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

ABSTRACTS

of Some of the Papers Read at the Fall Meeting

November 18 – 19, 1960

Galveston, Texas

Staphylococcus Killing Patterns for Chick Embryos. Sanders T. Lyles and Sonya West, Texas Christian University, Fort Worth, Texas.

Staphylococcus killing patterns for nine day chick embryos were tested by inoculation of approximately 10^6, 10^4, and 10^2 cells of each strain into the chorio-allontoic sac of the embryos. Five embryos were inoculated with each dilution, and candling performed at 24, 48, and 120 hours. The stocks tested, with one exception, were furnished by the University of Texas Southwestern Medical School at Dallas, Texas.

Seven strains from each of phage groups I and II, nine from phage group III, one from group IV, four untypables, and one coagulase negative strain have been employed in preliminary tests. Killing patterns varied considerably within the phage groups, as well as among the untypable coagulase positives. The coagulase negative and seven of the typable strains possessed negligible killing potency for the embryos. In general, the per cent of embryos killed by the strains from group II was considerably lower than that found by either group I, group III, or untypable strains.

Considerable hemorrhage and liver distortion usually accompanied death of the embryos. Further tests have not been completed, but are in progress.

A Study of Freund and Freund-type Adjuvants in the Production of Experimental Allergic Encephalomyelitis. W. J. Fahlberg, E. C. Alvord, C. M. Shaw and M. J. Kies, Baylor University College of Medicine, Houston, Texas; University of Washington Medical School, Seattle, Washington; and Laboratory of Clinical Science, N.I.M.D.B., Bethesda, Md.

During the course of experiments studying Experimental Allergic Encephalomyelitis in guinea pigs, it was observed that certain optimal ratios existed between the amount of antigen and the amount of Mycobacteria employed in Freund's adjuvant to induce maximal disease. The antigens studied were whole guinea pig cord and active encephalitogenic fractions of bovine spinal cord and the Mycobacteria were *Myc. tuberculosis* and *Myc. butyricum*. High levels of Mycobacteria (4 mg) inhibited the encephalitogenic activity of the antigens (.06–1.0 mg). Low levels of Mycobacteria (0.2 mg) were effective in inducing disease only when mixed with high levels of the antigens (0.4–

1.0 mg). Curves expressing the relationship between Mycobacteria and antigen in the induction of disease appeared diphasic. Preliminary evidence suggests this type of a curve is related to two different adjuvant activities of the Mycobacteria. One is contained in the cell wall and the other in the Wax "D" fraction.

Survival of Mammalian Cells (Strain HeLa) After Single and Fractionated Doses of X-rays. R. Z. Lockart, Dept. of Bacteriology, The University of Texas, Austin.

Several subcultures of S3HeLa cells of human origin were studied to determine their response to single and fractionated doses of X-rays. Survival curves generally displayed a threshold which suggested that accumulated damage was necessary before a lethal response occurred. The presence of a threshold was used as an indicator to show whether cells which had received a prior dose of X-rays still retained that damage. HeLa S3 cells were given an initial dose of X-rays sufficient to reach exponential survival (10 per cent survivors), allowed 24 hours incubation, then radiated further. Complete survival curves of the initial unirradiated cells and those which had received an initial dose both displayed a threshold. This indicated that accumulated events or damage as a result of X-rays had been lost by 24 hours. The magnitude which the disappearance of prior irradiation effects had on the fraction of cells surviving a given dose when given as several fractionated doses was discussed. Also, one subculture of HeLa cells, after radiation responded to a second dose of X-rays with a decreased average sensitivity as displayed by the slope of the exponential decrease in survivors.

Occurrence of Proteus *in Captive* Macaca mulata. Anna C. Garner, Microbiology-Cellular Biology Branch, School of Aviation Medicine, USAF Aerospace Medical Center (ATC), Brooks Air Force Base, Texas.

Proteus morgani has been reported as the etiological agent in sporadic cases of enteritis in monkeys. Rectal swab cultures from 264 *Macaca mulatta* were examined for this organism, as well as other species of *Proteus*. *P. mirabilis* was detected in 177, *P. morgani* in 78, *P. rettgeri* in 2, and *P. vulgaris* in 1. Direct plating to SS agar (76 positives) was far superior to selenite-F enrichment broth (3 positives) for the isolation of *P. morgani*.

Aerobacter-Shigella *Antagonism: Survey of 264* Macaca mulatta and *in vitro Studies of Isolates.* Anna C. Garner and E. Staten Wynne, Microbiology-Cellular Biology Branch, School of Aviation Medicine, USAF Aerospace Medical Center (ATC), Brooks Air Force Base, Texas.

There are several reports in the literature of antagonism of *Aerobacter* toward *Shigella*. Since shigellosis is an important disease in *Macaca mulatta*, investigations were conducted with this primate on the possibility of *in vivo* antagonism between these two genera. Examination of fecal specimens of 264 monkeys revealed *Shigella* but not *Aerobacter* in 4, *Aerobacter* but not *Shigella* in 16 and both genera in 1. However, in the animal with both organ-

isms, the ratio of *Shigella* to *Aerobacter* was considered so high (150:1) as to preclude effective antagonism. The low incidence of both genera resulted in the lack of coexistence not reaching statistical significance. However, occurrence of *in vivo* antagonism was rendered plausible by strong *in vitro* antagonism in liquid cultures between all 17 *Aerobacter* isolates and all 5 *Shigella* isolates.

Measurement of the Mouse Protective Effect of Antibody for Pseudomonas aeruginosa *in Burned and Unburned Patients' Sera.* C. D. Graber and D. Cummings, Surgical Research Unit, Brooke Army Medical Center, Ft. Sam Houston, Texas.

Native hemagglutinin to a pseudomonad polysaccharide (PC-9) present in the sera of burned and non-burned patients was shown to have little protective function for mice infected with a highly virulent nosocomial *Pseudomonas aeruginosa* phage type.

Study of sequential sera of a single burned patient surviving long enough to produce hyperimmune antibody to his pseudomonad septicemia suggests that immune bacterial agglutinin and immune hemagglutinin are protective. Hyperimmune sera from an unburned rabbit further substantiated this relationship.

A Comparison of Methods to Eliminate Nonspecific Fluorescent Antibody Staining. Susan J. Allen and Paul Donaldson. Department of Microbiology, The University of Texas Southwestern Medical School, Dallas.

Portions of fluorescein conjugated rabbit anti-human serum globulin were treated respectively by absorption with liver powder, by addition of 1/20th part of rhodamine conjugated normal calf serum, by fractionation with a diethylaminoethyl-cellulose chromatographic column, and by the addition of the rhodamine conjugated calf serum to the selected chromatographic fraction. Frozen sections were cut of the liver of a mouse sacrificed during anaphylactic shock involving human serum as antigen. These were stored seven weeks at both 0 and 25 C, both over drierite and in slide boxes, and in a sealed container at —20 C. These sections stained with the fluorescent antiserum portions were observed by ultraviolet illumination for specific staining of the antigen-containing Kupffer cells and nonspecific staining of the parenchyma. Sections stored at 0 C over drierite showed minimal nonspecific staining of the parenchyma and good specific fluorescence of antigen in the Kupffer cells, especially with the chromatographic fraction alone and this fraction combined with rhodamine coupled calf serum. Little specific or nonspecific staining of sections stored at 25 C was seen. Storage at —20 C produced results similar to those of 0 C storage.

Effect of Vibration and Temperature on the Specific Reactive Capacity of Antiserum. Jimmie Lee Flume, Immunology Section, Microbiology-Cellular Biology Branch, School of Aviation Medicine, USAF Aerospace Medical Center (ATC), Brooks Air Force Base, Texas.

The effect of heat and shaking on the specific reactive capacity of antiserum was investigated by both precipitation and passive hemagglutination. Results

indicate that, while the capacity of the antiserum to precipitate with homologous antigen is destroyed by heat, the antiserum is still capable of causing agglutination of red blood cells sensitized with the antigen. Shaking appears to enhance the precipitability of the antiserum. It was postulated that there exist, in the heated preparations of antiserum, protein molecules of various degrees of denaturation which are detectable by passive hemagglutination, but are below the threshold of sensitivity of precipitation technics. Enhanced precipitability after shaking might be attributed to the uncovering of normally inaccessible reactive sites.

Temperature Induced Pigmentation in Serratia marcescens. Cora Lee Gott and Robert P. Williams, Department of Microbiology, Baylor University, College of Medicine, Houston.

Serratia marcescens strain Nima doesn't form pigment at 38°C although adequate growth of cells occurs. Cultures incubated at 38°C, then transferred to 27°C will pigment provided the 38°C incubation period didn't extend beyond a critical time. The time differed between complete and minimal media. Cultures grown 48 hr. in minimal medium at 38°C wouldn't form pigment at 27°C. If casein hydrolysate is added to minimal medium after transfer to 27°C pigmentation occurred. Cells grown at 38°C, washed, and suspended in saline containing casein hydrolysate also formed pigment at 27°C. Measurement of protein and viable count established that neither increased under these conditions. Pigment formation was slower and reached only about one-half the concentration of growing cultures. Yeast extract didn't support pigment formation, but vitamin-free casein hydrolysate and a mixture of amino acids did. The optimum concentration of casein hydrolysate for pigmentation was 0.4 per cent. The optimum temperature was 27°C. Extreme acid or alkaline conditions prevented pigmentation. Temperature induced pigmentation provides a system in which growth and pigment formation can be separated.

An Improved Medium for Pyocyanine Production by Pseudomonas aeruginosa. Walter Sellers, E. Staten Wynne, and C. D. Graber, Microbiology-Cellular Biology Branch, School of Aviation Medicine, USAF Aerospace Medical Center (ATC) Brooks Air Force Base, Texas; and Brooke Army Medical Center, Fort Sam Houston, Texas.

For the rapid production of maximum amounts of pyocyanine by *Pseudomonas aeruginosa*, a culture medium composed of Noble's agar 2% (Difco), neopeptone 2% (Difco), sodium citrate 1%, and glycerol 1% has been shown to be superior to other media, including Sabouraud's Maltose Agar. Twenty stock culture strains and 39 fresh clinical isolates of *P. aeruginosa* were used in the study.

Growth Studies on the Cotton Root-rot Fungus, Phymatotrichum omnivorum *III. Assay of Growth-Promoting Substances.* Ralph J. Hervey, Texas Agricultural Experiment Station, Temple, Texas.

Phymatotrichum omnivorum, the Texas root-rot fungus, reproduces vegetatively only. It proliferates only from intact mycelial masses above a critical

size. An improved assay for growth-promoting substances has been developed through efforts to derive improved inoculum. Attempts to grow Phymatotrichum from washed, suspendible, mycelial fragments failed; 10 square m.m., nearly nutrient-free, water-agar-mycelium cores from uniform colonial matrices will proliferate. Phymatotrichum's ability to spread widely over nutrient-free agar, from small nutrient-bearing inocula provides basis of technique.

For assaying, cores are centered in Petri dishes containing 15 mls. of modified Roger's agar; 0.04 ml. of cold-sterilized material is applied on core. To determine lag-phase duration and quantity of initial growth (24 hours), core margins are examined microscopically through inverted dishes and number and length of radiating hyphae graded with increasing plus signs.

Subsequent determinations, *i.e.*, radiating-hyphae length (m.m.) and colony-matrix thickness (.0—15+), are made on a mechanical stage-adapter holding inverted dishes. For realistic measurement of characteristically irregular-edged, open-matrix colonies, they are divided into quadrants. To characterize total growth, composite value: defined as product of hyphal elongation × matrix-thickness, is calculated.

The following basic-medium supplements showed marked growth promoting effects: concentrated carrot juice, penicillin 10–20 ppm, gibberellic acid 50 ppm, Kinetin 0.2 ppm and several Phymatotrichum extra-cellular fractions.

Microbiological Effects of Space Radiations. I. Preliminary Report. Irving Davis and Thomas L. Roberts, Astromicrobiology Section, Microbiology-Cellular Biology Branch, School of Aviation Medicine, USAF Aerospace Medical Center (ATC), Brooks Air Force Base, Texas.

The application of a microbial genetic marker to the space radiation problem was investigated. The B/r/Sd-4 strain of *Escherichia coli*, reverting from streptomycin-dependency to nondependency, was the biological system. The effects of anticipated pre- and post-flight environments upon this system were determined. Starting with 7×10^8 cells per ml., survival dropped one log in less than four days. This rapid decay rate resulted in too few surviving cells remaining to detect spontaneous mutants. Increasing the initial number of cells did not increase the number of surviving cells for post-flight analysis. Other factors in the biological system were studied. Rigid control of pre- and post-flight temperature at 5°C resolved the problem. Data from rocket and balloon space flights were presented.

Oxidation of Alkyl Substituted Cyclic Hydrocarbons by a Nocardia. J. B. Davis, Socony Mobil Oil Company, Inc., Dallas, Texas.

Nocardia 107–332, a soil isolate, oxidizes short-chain alkyl substituted cyclic hydrocarbons to cyclic acids while growing on *n-alkanes*. Cyclic acids are produced also from relatively long-chain alkyl substituted cyclics such as *n*-nonylbenzene or *n*-dodecylbenzene which alone support growth in a mineral-salts medium. Ω-oxidation of the alkyl substituents is followed by β-oxidation. It is of particular interest that cyclic acids such as cyclohexane-

acetic and phenylacetic with C_2 residual carboxylic acid subsituents are resistant to further oxidation by the nocardia but cyclic acids with C_1 or C_3 substituents are readily oxidized and utilized for growth.

The specificity of microbial oxidations is demonstrated by the conversion of *p*-isopropyltoluene (*p*-cymene) to *p*-isopropylbenzoic acid in *n*-alkane growth-supported nocardia cultures.

Azotobacter Bacteriophage. Orville Wyss and M. W. Nimeck, Department of Bacteriology, The University of Texas, Austin.

A series of one-step growth experiments in nitrogen-free minimal medium were undertaken for further characterization of ten bacteriophages for *Azotobacter vinelandii* strain O previously isolated in this laboratory. No relation between serological grouping and latent periods or burst sizes could be demonstrated, except for two phages, A-21 and A-23, which were previously suggested to be identical.

No results could be obtained for phage A-21 in minimal medium although a burst of 70 was demonstrated in medium supplemented with 1% casein hydrolysate. The effects of supplementation with various phage/cell ratios, pH and chelation were investigated. The lack of increased titres with the addition of simulated casein hydrolysate, mixed B vitamins and inorganic nitrogen suggested that a simple co-factor or nitrogen supplement was not involved. Urea, which is utilized by non-nitrogen fixing mutants of the host organism did cause increased titres.

It is hoped that a rapid screening method might be developed to determine the nature of the bacteriophage requirement which may be associated with amino acid synthesis, nitrogen or carbohydrate metabolism.

Effect of Medium Composition on the Inhibition of Staphylococcal Phages. C. E. Dowell and E. D. Rosenblum, Department of Microbiology, The University of Texas Southwestern Medical School, Dallas, Texas.

Other authors had reported the inhibition of lysis of Group I staphylococcal typing phages by Brain Heart Infusion Agar (BHI). We found that the serologic Group B staphylococcal phages were inhibited on BHI while the serologic Group A phages were not.

Phosphates were implicated as the major inhibitory component of BHI. Phosphates added to a non-inhibitory medium such as trypticase soy agar could make it inhibitory, and when excess amounts of phosphate were added, the Group A phages were also inhibited.

Cells surviving infection by serologic Group B phage on BHI did not become lysogenic for the challenging phage.

BHI itself did not inactivate the phage particle. From a series of experiments in which Group B phage and cells were plated together on BHI and other media, we concluded that the primary effect of BHI was due to poor adsorption of phage. It was shown that cells were not rescued from lysis by plating on BHI after phage adsorption had occurred.

Anaplasma marginale—*A Parasite of Bovine Erythrocytes.* F. C. Heck, T. E. Franklin, J. W. Huff, Department of Veterinary Microbiology, Texas Agricultural and Mechanical College, College Station.

Anaplasmosis, a disease in cattle, is caused by the organism Anaplasma. The economic importance of anaplasmosis is evidenced by the 50 to 75 million dollar lost each year as a result of its prevalence. In the acute stage of this disease, small inclusion bodies can be observed in the erythrocytes of infected animals. These bodies are absent in blood smears from carrier animals. The carrier animal is detected on the basis of results from the complement-fixation test for the anaplasma antibody.

Transmission of Anaplasma is believed to be accomplished by blood sucking insects. However, contingent transmission by specific vectors remains speculative.

At the School of Veterinary Medicine, Texas A&M College, we have an active research program on this disease. Our efforts are directed to enable us to gather information on the taxonomy, ecology, etiology and physiology peculiar to this organism and the disease that it transmits.

Action of Oral Fusiform Bacilli on Ascorbic Acid. William A. Nolte and Maria Winterbotham, Department of Microbiology, The University of Texas Dental Branch, Houston, Texas.

Seven strains of oral fusiform bacilli isolated from normal and diseased mouths were grown 24 hours in Brewer's medium centrifuged, washed several times with saline and resuspended in 5 ml. of saline. One ml. suspension of each strain was inoculated into a modified micro inoculum broth without dextrose but with added ascorbic acid, 0.5 mg. per ml. of broth, and .05% agar. All cultures were incubated anaerobically under an atmosphere of 95% nitrogen and 5% carbon dioxide at 37°C. Ascorbic acid determinations were made at 24, 48 and 72 hour intervals using standardized Sodium 2,6-Dichlorophenolindophenol dye. Adequate uninoculated and inoculated controls were also tested.

Five of the seven strains designated as Group 2, reduced the concentration of ascorbic acid to nearly one half within 24 hours, and to approximately zero concentration by 48 hours. Two of the strains designated as Group 1, appeared to have no effect on this vitamin.

To determine the effect of dextrose and sucrose on utilization of ascorbic acid, 1% concentrations of each of these carbohydrates was added to the test medium with the added vitamin.

Results with Group 1 strains were the same for each carbohydrate as in the previous tests. Group 2 strains revealed a sparing action of both carbohydrates for ascorbic acid. Added dextrose showed some but not complete protection for the vitamin, whereas, sucrose gave apparently complete protection.

From the results of the seven strains tested, there appears to be no difference between strains of fusiform bacilli isolated from normal or diseased mouths and their ability to destroy ascorbic acid.

IN VITRO EFFECTIVENESS OF FURALTADONE ON ANTIBIOTIC-RESISTANT STAPHYLOCOCCI[a]

JOSEPH A. BASS[b]

The problem of treating staphylococcal infections due to strains resistant to the more commonly used antibiotics is well known. A search for new chemotherapeutic agents effective against such organisms has led to the introduction of a number of new drugs into clinical practice. Among those currently receiving clinical trial, furaltadone* appears to offer the advantages of oral administration and a low order of side effects.[1, 2] Preliminary tests in this laboratory have shown its uniform *in vitro* activity effectiveness against all strains of *Staphylococcus aureus*, freshly isolated from various lesions. These results, however, were based solely on sensitivity tests performed with 50 mcg. discs. In the present study, testing was performed with 10, 20, and 50 mcg. discs, as well as by the tube dilution method.

Materials and Methods

In a first group (Table 1), 194 strains of coagulase-positive, pigmented staphylococci, recently isolated from infections or from various wounds were tested. Because these strains were selected largely on the basis of their resistance to 2 or more of the generally used antibiotics, they do not represent accurately the resistance picture generally encountered. Previous studies,[3] however, indicated that approximately 50 per cent of all staphylococcal infections in the hospitals of the Medical Branch of the University of Texas were attributable to such strains.

A second group of strains tested (Table 2) consisted of 266 cultures isolated approximately one year previously, in the course of epidemiologic studies. All were coagulase-positive, and the majority produced a golden pigment.

The first group was tested against furaltadone and 6 antibiotics by the serial tube dilution technic (Table 3). Double dilutions of the agents prepared in trypticase soy broth were inoculated with one drop of a 1:1000 dilution of an 18-hour broth culture of the organism; tubes were read for

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[a] Department of Microbiology, Medical Branch, The University of Texas, Galveston. Paper accepted for publication Nov. 23, 1960. This work was supported in part by a grant from the Eaton Laboratories, Norwich, N.Y.

[b] The author expresses his appreciation to Mrs. Dvora Feigon Roth and to Mrs. Jo Anita Massa Jeffries for their technical assistance.

* Altafur, Eaton Laboratories.

TABLE 1

Sources of Strains of Staph. aureus
(Tested in First Group)[*]

Source	Phage Type 80/81	Other Typables	Non-Typables	Total
Boils, abscesses, wounds, post-operative infections, etc.	97	36	23	156
Trachea	3	..	2	5
Blood	2	..	1	3
Ear	1	3	2	6
Eye	3	1	..	4
Genitourinary tract	..	1	3	4
Throat, nasopharynx, sputum	5	6	5	16
Totals	111	47	36	194
Per Cent	57	24	19	..

[*] 194 freshly isolated strains.

TABLE 2

Sources of Strains of Staph. aureus
(Tested in Second Group)[*]

Source	Phage Type 80/81	Other Typables	Non-Typables	Total
Boils, abscesses, wounds, post-operative infections, etc.	54	16	11	81
Trachea	1	2	2	5
Blood	6	..	1	7
Ear	2	1	1	4
Eye	2	4	..	6
Genitourinary tract	1	1	5	7
Osteomyelitis	2	..	..	2
Anterior nares (carrier survey)	58	11	7	76
Nasopharynx (patients)	50	12	16	78
Totals	176	47	43	266
Per Cent	66	17	17	..

[*] 266 isolates obtained one year before.

TABLE 3

Tube Dilution Sensitivities of Staph. aureus
*(First Group Only)**

Minimal[a] Inhibitory Concentration	Per Cent Sensitive						
	Penicillin	Erythromycin	Tetracycline	Chloramphenicol	Novobiocin	Kanamycin	Furaltadone
50	77.3	34.5	13.9	1.5	..	1	..
50	9.3	2.1	73.2	9.8	0.5	2.6	..
25	6.2	2.1	8.8	6.7	16.0	25.3	0.5
12.5	4.1	3.1	2.1	5.7	13.9	25.8	9.3
6.25	..	1.5	..	28.4	7.2	21.6	81.4
3.12	..	2.1	..	33.5	6.2	17.5	8.8
1.56	0.5	4.1	..	10.8	8.2	4.6	..
0.78	0.5	3.1	0.5	2.6	8.2	1.5	..
0.39	0.5	3.6	1.0	1.0	13.4	..	..
0.19	1.5	43.8	0.5	..	26.3	..	..

* 194 strains.
[a] mcg. or units/ml.

turbidity following overnight incubation at 37 C. Broth groups were also tested for susceptibility to furaltadone by the disc method.

Results and Conclusions

By the tube method (Table 3), almost all strains of the first group were resistant to 6.25 units/ml. of penicillin (97%) and tetracycline (98%), with the following in decreasing order: kanamycin (55%), erythromycin (42%) and chloramphenicol (24%). On the other hand, only 10% of the strains tested were resistant to 6.25 mcg./ml. of furaltadone. All strains in this group were susceptible to furaltadone when tested by the disc method with 10, 20, and 50 mcg. discs. Similarly, all the strains in the second group were sensitive by the disc technic (only 50 mcg. discs were used with this group). In all instances inhibition zones were sharp, with no evidence of secondary growth occurring within the primary zones, even after plates had been incubated for several days.

Studies in humans show that with half the recommended furaltadone dose (800 to 1000 mg./day), plasma levels reach a peak of 5.2 mcg./ml. in 4 hours, decreasing gradually to 2 mcg./ml. in 8 hours.[4] Protein binding of this drug is estimated at 20%, and presumably this is a very loose binding, since in

dogs the plasma and cerebrospinal fluid concentrations are comparable.[4] Inasmuch as serum levels are in the general range of concentration necessary for inhibition of 90% of multiple resistant strains of staphylococci *in vitro*, it would appear justifiable to consider furaltadone as a suitable adjunct in the treatment of infections due to these organisms.

However, results obtained with furaltadone by means of the disc test must be viewed with caution, since inhibition was uniformly obtained with 10 mcg. discs, even though 10% of the 194 strains in the first group were resistant to 6.25 mcg./ml. These results suggest the need for further comparative tube dilution and disc susceptibility studies, to determine whether the concentration of furaltadone in sensitivity discs should be lower than those employed presently.

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MALPOSITION OF THE HINDGUT. REPORT OF TWO CASES[a]

A. R. Bhadkamkar and V. R. Mysorkar

As stated by Cherney, "Statistics indicating the frequency of malposition of the gut are understandably inaccurate." (3). According to Dott "error in location of the attachment of the hindgut is uncommon. The splenic flexure is very constant in location. Variations in the course of the descending and sigmoid colons constitute but minor modifications of their normal curves or degree of adhesion. Black has reviewed the subject and finds only nine records of cases in which the descending colon curved so far in its course as to reach the midline. The sigmoid colon is liable to great variation in its length and hence in position, but its primary attachment is remarkably constant." (4). Bhattachayya (2) found three cases of right sided sigmoid colon among fifty-nine bodies dissected during 1923–4. Dubey (5) reports a case of anomalous descending colon in which a major part of the descending and sigmoid colons were transposed. Sheinfeld (6) reports one case of right sided sigmoid colon, which was involved in a carcinoma of the caecum.

According to us, cases of malposition of the descending colon are very rare. One of us (A.R.B.) has found only three cases of this type, one of which was Situs Inversus Totalis (1), and the other two which will be described below, in over 800 bodies dissected in this department during the last 12 years. It is felt that the finding of these cases of right sided descending colon by Bhattachava in as few as 59 bodies is a chance coincidence. Our cases are interesting because in one, the major part of the descending colon and the rest of the colon below, was transposed and fixed in that position. In the other case the primary attachment of the sigmoid colon was not in its normal position, it being fixed in the transposed position and the rectum below deviated to the right.

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Case Reports

Number one. This is the body of an adult male found at the roadside. Cause of death is unknown. The abdomen has no free fluid. The clils of the small bowel appear to be crowded to the left half of the abdomen. The caecum is distended and lies midway between the inferior surface of the liver and right iliac fossa. The ascending colon is very short, about 1½ inches in length. The appendix is retrocaecal. The transverse colon is 9 inches long and normally placed. The splenic flexure is normally placed. The descending colon descends vertically for about 3 inches and then deviates almost at right angles toward the right side to cross the disc between 3rd and 4th lumbar vertebrae, crosses behind the superior mesenteric vessels and reaches the right lumbar region to continue as the sigmoid colon. The sigmoid mesocolon is short, its attachment from the right lumbar region to the right side of the third sacral vertebra crossing the bifurcation of the right common iliac artery. The rectum is normal. The whole descending colon is retroperitoneal, and has no mesentery. The caecum appears to rest on the junction of the descending and sigmoid colons. The length of the descending colon is roughly 12 inches and the sigmoid colon 6 inches. Other abdominal and thoracic viscera normal.

Number two. The body is that of an adult male found at the roadside. Cause of death unknown. There is no free fluid in the abdomen. The caecum lies in the right iliac fossa. The appendix is fibrosed and two inches long. The ascending colon is four inches long, transverse colon nine inches long and splenic flexure normal. The descending colon courses vertically to the left iliac fossa, crosses the left external iliac artery, bends to the right, ascends, crosses the body of the fifth lumbar vertebra, to enter the right iliac fossa behind the caecum which is freely movable. The colon then forms a loop, crosses the right external iliac artery and reaches the midline of the body near the upper end of the sacrum. The rectum is not in the midline and cannot be demarcated from the sigmoid loop. The whole of the hindgut distal to the splenic flexure is retroperitoneal. The length of the colon distal to the splenic flexure is 27 inches. The rest of the abdominal and thoracic viscera are normal.

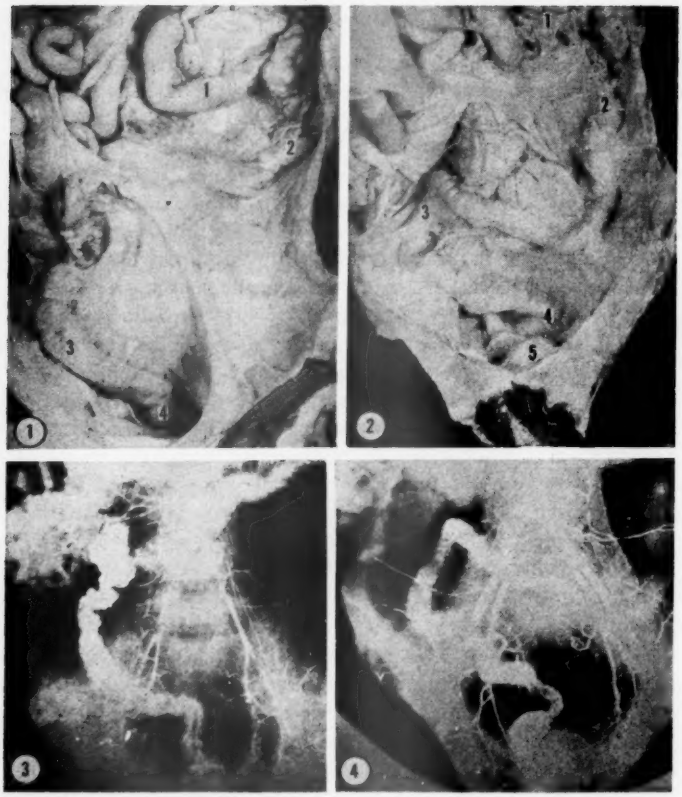
Discussion

No satisfactory explanation for malposition of the hindgut has been afforded. Bhattachayya feels that there is secondary rotation of the large gut while primary rotation is taking place and could cause such an anomaly. Our explanation is different. Before the rotation of the intestines the gut is suspended from the dorsal body wall by a midline mesentery. During the second stage of the gut rotation, while the caecum, adjacent ileum and colon are still in the umbilical cord, the coils of the small bowel return to the abdomen at the right side of the superior mesenteric artery, which is suspended along the abdomen between its origin from the aorta and the umbilicus. Normally these coils, after occupying the available space in the right half of the abdomen, later push the hindgut and its mesentery, which occupies the midline, to the left. At this time, if the hindgut has an abnormally short mesentery, it is possible that these loops may enter the abdomen in front of the hindgut, and as they fill the space on the left side, push the hindgut toward the right, thus accounting for the findings in case one.

The third stage of rotation ends in the fixation of some of the abdominal viscera or parts of the intestine to the posterior abdominal wall, or to each other, resulting in the loss of their mesentery. In our first case the hindgut below the splenic flexure appears to be fixed in this malrotated position. In our second case it appears that the elongated sigmoid loop underwent a second rotation before its mesentery became fixed to the left side and fixation occurred in the abnormal position after secondary rotation.

Incomplete descent of the caecum is usually attributed to early fixation of the caecum or to failure in elongation of the colon. In our first case it appears that the caecum could not descend completely and the abnormally located descending colon occupied the right iliac fossa and consequently the ascending colon is very short. In the second case the caecum is not fixed to the posterior abdominal wall, as the abnormal sigmoid loop already occupied the right iliac fossa and is freely movable.

It does not appear that such abnormalities necessarily have any bearing on the cause of death.



FIGS. 1 and 3. Disposition of viscera in case one and skiagram of the same after barium enema and arterial injection.
FIGS. 2 and 4. Case two treated as above. 1, Transverse colon, 2, Descending colon, 3, Sigmoid colon, 4, Rectum, 5, Urinary bladder.

Two interesting cases of malposition of the hindgut are reported. A probable explanation for these abnormalities has been attempted.

Acknowledgments

We sincerely thank Dr. B. M. Desai M.S.; F.C.P.S.; Professor of Anatomy, for permitting us to publish these cases. Our thanks

are also due to Mr. V. N. Joshi, our College Artist, for the photographs.

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OBSERVATIONS ON THE MODE OF ACTION OF AN OROTIC ACID ANTAGONIST, N-OROTYL-N′ N′ - DIETHYL-ETHYLENEDIAMINE HCl,[a] ON CELLS GROWN IN TISSUE CULTURE

JOHANNA BLUMEL[b] AND JOHN KOK[c]

Introduction

Orotic acid has been shown to be a pyrimidine precursor. Under the influence of diphosphopyridine nucleotide, reduced, it is converted into dihydro-orotic acid which is phosphorylated and decarboxylated to a uracil monophosphate. This uracil monophosphate may then be incorporated into ribonucleic acid.

Several compounds that seem to act as antagonists by inhibiting the phosphorylation of orotic acid have been synthesized. In the present investigation a new derivative of orotic acid, N-orotyl-N′ N′-diethyl-ethylenediamine HCl (ODED) is employed. The antagonistic activity of the compound is thought to be brought about by blocking the orotic acid phosphate decarboxylase.

Materials and Methods

Tissue Cultures: Four well established tissue culture cell lines were utilized in this investigation, (1) HeLa strain, (2) Fernandes Human Amnion strain, (3) Chang's Conjuctiva strain and (4) McCoy's Synovium strain. In addition, a smoke condensate-induced tumor of a C_3H mouse which was diagnosed as a Leiomyosarcoma (Mizutani *et al.* 1960) was also employed. Only results from the HeLa strain will be reported in this publication.

The tissues were cultivated by the cover slip and Rose chamber methods, using 25,000 cells per chamber with Eagles medium as the nutrient. After three days growth a sufficient number of cultures were treated with the chemical agents which were dissolved directly in the medium and added to the cultures. The chemicals had been tested for toxicity. Control cultures were kept on Eagles medium.

Procedure: Three day old Rose chamber preparations of HeLa cells were divided into four groups and treated as follows: Group 1 was treated with

[a] The N-orotyl-N′ N′—diethyl-ethylenediamine HCl was kindly furnished by Mr. C. U. Kloosterman, Central Laboratory, Noury and v. d. Lande, Deventer, Holland.

[b] From the Department of Surgery, Division of Orthopedics, and the Tissue Culture Laboratory, The University of Texas Medical Branch, Galveston, Texas. Paper accepted Nov. 28, 1960.

[c] From the Biochemical Laboratory, University of Amsterdam, Amsterdam, Holland.

5 mg/ml ODED. Group 2 was treated with 2.5 mg/ml thymine. Group 3 was treated with 5 mg/ml ODED plus 2.5 mg/ml thymine. Group 4 was used as the control. Media were replaced on all cultures whenever changes were made on any group.

After 24 hours, ODED treated and control cultures were subjected to time-lapse cinematography. In addition some of the group 1 treated preparations were continued on ODED, some had the chemical replaced with plain medium, some had it replaced with thymine and some were treated with ODED plus thymine. One-half of the cultures in group 2 were continued on thymine. The remaining cultures were replaced with Eagles medium. The group 3 cultures were continued as before. After one and three days growth, phase contrast photographs, Plates 1, 2, and 3 were taken. All of the preparations were fixed after another 24 hours growth.

Staining: Preparations of all groups were stained by the Feulgen, PAS, Sudan black, Jacobson and Acridine orange methods. The latter for visualization by fluorescence microscopy.

Results and Discussion

Plates 1, 2 and 3 show living HeLa cells as seen by dark phase microscopy after five and seven days growth under various conditions. In plate 1 there is no change in the cultures after one and three days treatment. All changes have occurred within 24 hours after treatment. Control cells grown in Eagles medium (fig. 1). HeLa cells after two and four days treatment with ODED and thymine respectively (figs. 2–3). Cultures treated with thymine for 24 hours and then replaced with Eagles medium for one and three days (fig. 4).

Plate 2 shows a four day old culture treated with ODED for one day and replaced with Eagles medium for one day (fig. 5). The same culture (fig. 6) after three days replacement with Eagles medium. The vacuoles observed in ODED treated cultures (fig. 2) have disappeared almost completely after one day replacement with Eagles medium (fig. 5) and on the third day the culture (fig. 6) has recovered and looks like the control (fig. 1). Cells treated with ODED for one day and replaced with thymine for one day (fig. 7). The same culture (fig. 8) after replacing the thymine with Eagles medium for one day. It will be noted that the cells have regained their normal appearance. It was found that the ODED treated cells replaced with thymine (fig. 7) recovered more rapidly than ODED treated cells replaced with Eagles medium (fig. 6). The cells (fig. 7) are very similar to those treated with thymine for two and four days (fig. 3).

When cells were treated with ODED for one day and then had

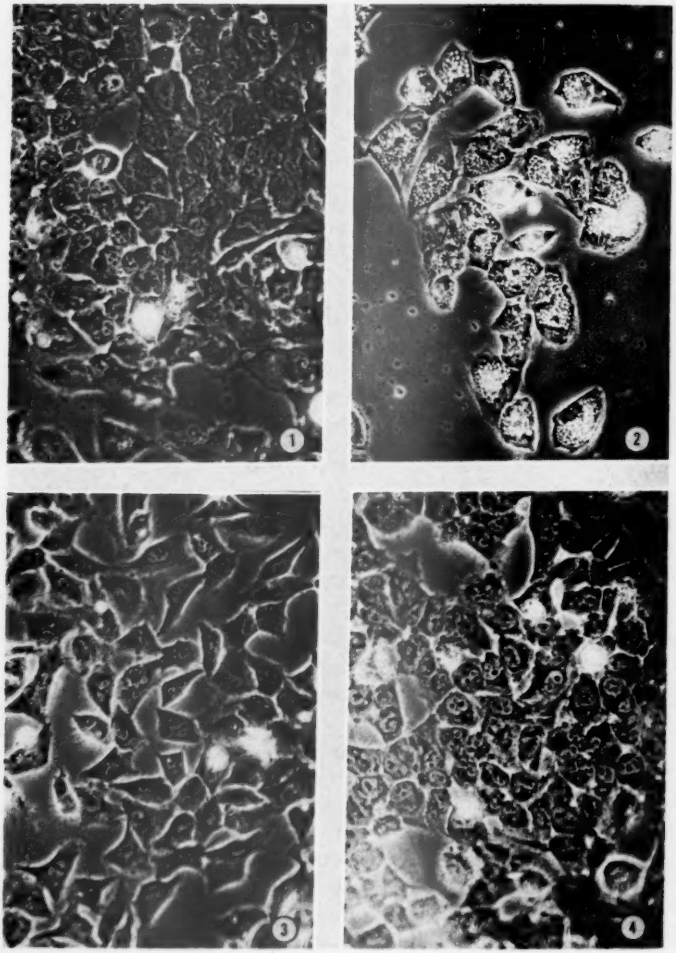

PLATE 1

FIGS. 1–11: Living HeLa cells as seen by dark phase microscopy after five and seven days growth under various conditions.

FIG. 1: Control cells grown in Eagles medium. No difference in photographs taken two days apart.

FIG. 2: Three day old cells treated with ODED for two and four days. No difference in photographs taken two days apart.

FIG. 3: Three day old cells treated with Thymine for two and four days. No difference in photographs taken two days apart.

FIG. 4: Three day old cells treated with Thymine for one day and then replaced with Eagles medium for one day. No difference in photographs after one and three days treatment.

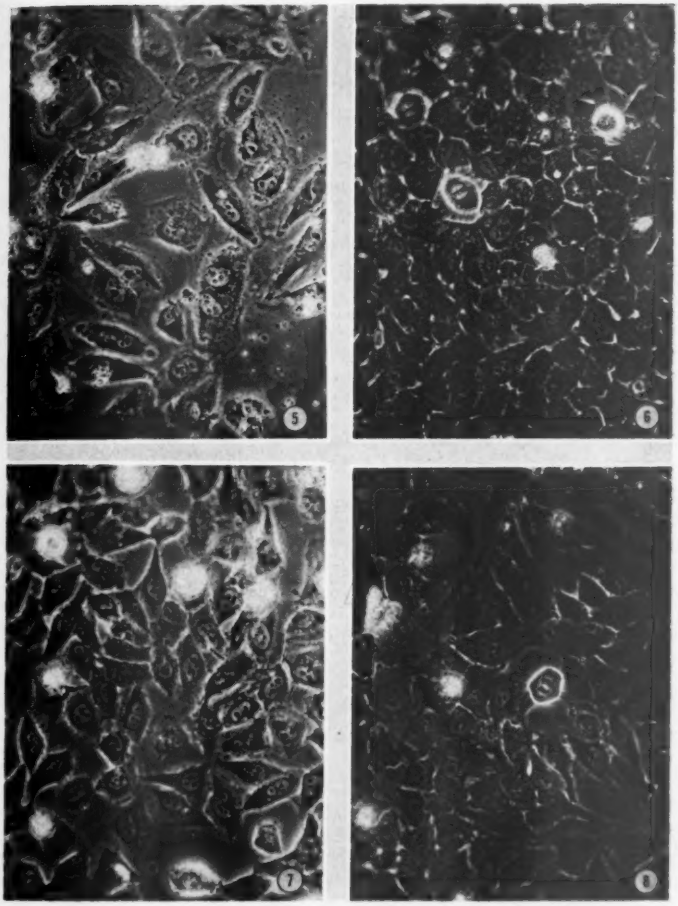

PLATE 2

FIG. 5: Three day old cells treated with ODED for one day and then replaced with Eagles medium for one day.

FIG. 6: Same as FIG. 5 after three days replacement with Eagles medium.

FIG. 7: Three day old cells treated with ODED for one day and then replaced with Thymine for one day.

FIG. 8: Three day old cells treated with ODED for one day, then replaced with Thymine for one day and then replaced with Eagles medium for one day.

thymine added for one day (Plate 3, fig. 9) the cells suffered greater damage. After this culture was treated with Eagles medium for one day the cells, (fig. 10) were unable to recover. Cultures treated with ODED for one day and then replaced with thymine for one and three days (fig. 11). It will be noted that the culture in this instance shows more damage than the culture where thymine was added to the ODED (fig. 9). In the group where ODED and thymine were used to treat three day old cultures the cells were completely irradicated after two days treatment.

Summary and Conclusions

From the photographs and stained preparations it appears that ODED, in the concentrations used, inhibits normal cell division of the HeLa strain. The mode of action is very similar to that of radio-active substances. Thymine counteracts the cytostatic activity produced by ODED. After replacement of ODED with thymine the cells recover very rapidly (fig. 7). Replacement of ODED with Eagles medium only is also followed by recovery, though more slowly (figs. 5–6). ODED appears to be a true pyrimidine antagonist with low toxicity.

Although these results seem to support the theoretical possibilities, further studies are necessary to determine the usefulness of ODED as an anti-carcinogenic agent. A complete report of the experiments will be published soon.

Acknowledgment

The authors wish to thank Dr. C. M. Pomerat and the staff of the Tissue Culture laboratory for technical assistance and the use of the facilities for this investigation.

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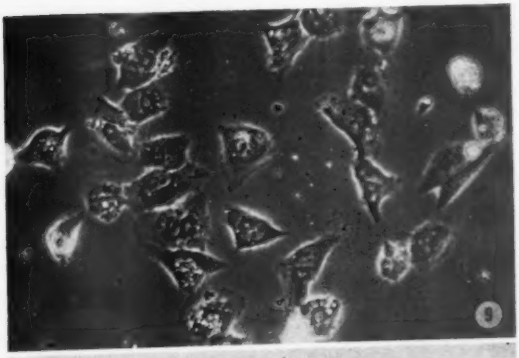
PLATE 3

FIG. 9: Three day old cells treated with ODED for one day, then Thymine added for one day.

FIG. 10: Three day old cells treated with ODED for one day, then Thymine added for one day and then replaced with Eagles medium for one day.

FIG. 11: Three day old cells, ODED treated for one day and then replaced with Thymine for one and three days. No difference in photographs taken two days apart.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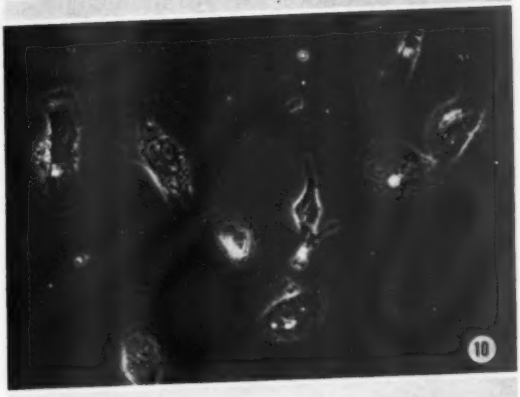
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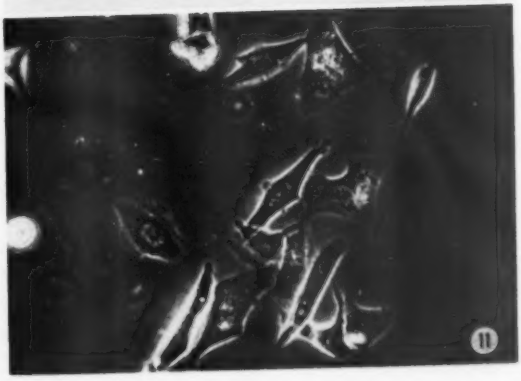
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THE EFFECT OF E-AMINOCAPROIC ACID ON FIBRINOLYSIN AND ON ACTIVATORS OF PROFIBRINOLYSIN[a]

D. R. CELANDER, MARY DELL NASCHKE, AND M. M. GUEST[b]

Attention has recently been directed to the clinical evaluation of certain commercial products reported to be of value in the treatment of intravascular coagula (10, 12, 29). As a consequence, interest has developed in a compound, epsilon-aminocaproic acid (E-ACA), which was reported a number of years ago to inhibit fibrinolysin (22). Several papers have recently appeared which have laid heavy emphasis on the role of E-ACA as an inhibitor of activators (5, 6, 16, 24, 31) with particular emphasis on the activator in plasma generated by streptokinase.

The impression of the predominant anti-activator activity of E-ACA has been further strengthened by the finding that E-ACA could be used *in vivo* to inhibit the action of urokinase in the genito-urinary tract (20).

In this paper are presented the results of *in vitro* studies employing three different mammalian systems which clearly indicate that E-ACA not only acts as an anti-activator but also possesses strong antifibrinolytic characteristics.

Materials

Canine profibrinolysin was isolated, converted to fibrinolysin, and assayed as directed by Celander and Guest (8). The product, when fully activated, contained 1 Celander unit of canine fibrinolytic activity per mg. protein (8).

Bovine profibrinolysin was prepared essentially as directed by Loomis *et al.* (18). It was converted by human urokinase to fibrinolysin. Assay by the method of Guest *et al.* (15) revealed that the profibrinolysin yielded 0.1 unit of fibrinolytic activity per mg. protein. Chloroform-activated fibrinolysin (bovine) was generously supplied by E. C. Loomis of Parke, Davis and contained 0.14 Guest units/mg.

Human profibrinolysin was precipitated from plasma essentially as directed

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[b] From the Department of Biochemistry and the Carter Physiology Laboratory, University of Texas Medical Branch, Galveston, Texas. Accepted for publication November 23, 1960.

by Loomis *et al.* (18) for the preparation of bovine profibrinolysin. It was converted to fibrinolysin by urokinase and possessed, when fully activated, about 0.1 Guest units/mg. protein (15). A preparation of human profibrinolysin generously supplied by Dr. Daniel Kline was also employed as a source of fibrinolysin; it contained approximately 25 Remmert-Cohen units/mg. N (28). This same preparation was spontaneously activated by incubation in 50% glycerol (1 mg. profibrinolysin/ml. 50% glycerol) (4) for 30 days at room temperature and found to contain 0.3 Guest units/mg. (15).

Armour's bovine fibrinogen, the protein of which was about 80% clottable, was dissolved in imidazole-saline buffer, diluted and used undialyzed. Freeze-thaw bovine fibrinogen was prepared and assayed essentially as directed by the method of Ware *et al.* (32) and contained 98 to 100% clottable protein.

Staphylokinase was prepared essentially as described by Lewis and Ferguson (17). The highly active preparation was used at a concentration of 0.02% in the incubation mixtures.

Streptokinase, was generously supplied as Varidase®, by Dr. E. G. Ruegsegger of Lederle Laboratories, in vials containing 100,000 units of streptokinase (11) and 25,000 units of streptodornase.

Purified urokinase, prepared and standardized by the method of Celander and Guest (9), contained about 2000 activator units/mg. protein.

Thrombin, Topical, was supplied through the courtesy of Dr. Cy Campbell of Parke, Davis in vials containing 5000 units.

The epsilon-aminocaproic acid (E-ACA) employed was generously made available to us as a white crystalline powder by Dr. E. G. Ruegsegger of Lederle Laboratories and by Dr. Bütler of the Swiss Red Cross through arrangements made by Drs. F. K. Beller and E. F. Luscher.

The buffers employed were 0.15 M Tris (13), pH 7.4, and imidazole buffer (21), pH 7.25. Imidazole-saline buffer was prepared by mixing 1 part imidazole buffer with 9 parts 0.9% saline.

Experimental and Results

Two-stage assay of activator inhibitor potency of E-ACA. The inhibitory effect of E-ACA on staphylokinase and urokinase was determined in a two-stage assay system. In the first stage, 0.4 ml. of varying amounts of E-ACA, dissolved in Tris buffer, was added to 3.6 ml. of imidazole buffer containing 16.4 Celander units of dog profibrinolysin (8), 0.44 Guest units of bovine profibrinolysin (15), or 1.44 Guest units of human profibrinolysin (14) and 2 units of human urokinase (9) or 0.8 mg. staphylokinase. The various mixtures were incubated at 37° C.; 0.1 ml. aliquots were withdrawn at 10 minute intervals for the first hour and at 20 minute intervals thereafter. The total time of incubation ranged from 70 to 300 minutes. The fibrinolytic activity generated was estimated in the second stage of the pro-

cedure which consisted of the determination of the lysis time at 37° C. of a clot produced by approximately 0.6 unit of bovine thrombin in a system containing the 0.1 ml. aliquot of incubation mixture, 0.1 ml. 0.9% saline, and 0.2 ml. 0.2% Armour's or "freeze-thaw" bovine fibrinogen.

The data are presented in Tables 1, 2, 3.

Determination of the effect of E-ACA on fibrinolytic activity. The 0.4 ml. test mixtures were prepared in 10 × 75 mm. test tubes by adding in order: 0.2 ml. 0.2% bovine fibrinogen, 0.05 ml. 0.9% saline; 0.05 ml. Tris buffer containing varying amounts of E-ACA, and 0.1 ml. of fibrinolysin solution. The final concentration of E-ACA in the clot ranged from 6.25×10^{-6} M to 1.25

TABLE 1

Inhibition of staphylokinase activation of canine profibrinolysin by ε-aminocaproic acid (two-stage system)

Incubation time (minutes)	0	Concentration of E-ACA in incubation mixture 5×10^{-4} M	1×10^{-3} M	5×10^{-3} M	1×10^{-2} M*
		Centiunits Fibrinolysin/ml.			
0	0	0	0	0	0
60	380	152	540	0	0
120	410	520	740	116	0
		Per Cent Change from Control Values			
60	..	— 60	+ 42	—100	—100
120	..	+ 27	+ 81	— 72	—100

* While the figures given for E-ACA of 1×10^{-2} M indicate 100% inhibition at 60 and 120 minutes, five hours later 94 centiunits fibrinolysin/ml. could be demonstrated in the incubation mixture.

TABLE 2

Inhibition of human urokinase activation of bovine profibrinolysin by ε-aminocaproic acid (two-stage assay)

Incubation time (minutes)	0	Concentration of E-ACA in incubation mixture 5×10^{-4} M	1×10^{-3} M	5×10^{-3} M
		Centiunits Fibrinolysin/ml.		
10	7.2	6.7	6.0	5.2
50	10.8	12.0	11.4	6.4
		Per Cent Change from Control Values		
10	..	— 7	—17	—28
50	..	+11	+ 6	—41

$\times 10^{-2}$ M. Centiunits of fibrinolysin contained in the test clots were: canine, 44; bovine, 0.9; and human, 1.24. Clot formation was induced by the immediate addition of approximately 0.6 units thrombin. Clot lysis was determined by the "tilt-tube" method of Guest (14) and expressed in the appropriate units (Table 4).

One-stage determination of anti-activator potency of E-ACA. This test was designed to measure the effect of E-ACA on the activator which is developed in human and dog plasma in the presence of streptokinase and a source of profibrinolysin. The 0.4 ml. clot employed contained 0.2 ml. 0.2% Armour's bovine fibrinogen, 0.05 ml. canine plasma diluted 1/50 or human plasma

TABLE 3

Inhibition of human urokinase activation of human profibrinolysin by ε-aminocaproic acid (two-stage assay)

Incubation time (minutes)	Concentration of E-ACA in incubation mixture			
	0	1×10^{-3} M	5×10^{-3} M	1×10^{-2} M
	Centiunits Fibrinolysin/ml.			
50	36	28	9	5.6
120	21	24	10	4.8
	Per Cent Change from Control Values			
50	..	—22	—75	—85
120	..	+14	—52	—77

TABLE 4

Effect of ε-aminocaproic acid on activity of Fibrinolysin

E-ACA concentration	Fibrinolysin: source and concentration		
0	Canine 44*	Bovine 0.92*	Human 1.24*
	Per Cent Change from Control Values		
6.25×10^{-6} M	0	— 5	—10
1.25×10^{-5} M	— 7	— 9	0
6.25×10^{-5} M	0	—17	+ 1
1.25×10^{-4} M	— 9	—20	+ 1
6.25×10^{-4} M	—44	—53	—70
1.25×10^{-3} M	—64	—71	—72
6.25×10^{-3} M	—94	—90	—78
1.25×10^{-2} M	—94	—99.5	—83

* Concentration of fibrinolysin is expressed in terms of centiunits (1/100 unit) fibrinolytic activity per 0.4 ml. clot. See References 8, 14 and 15 for unit definitions.

diluted 1/3200 (equivalent to 1×10^{-3} ml. dog plasma or 1.6×10^{-5} ml. human plasma per 0.4 ml. clot), 0.05 ml. of varying concentrations of E-ACA dissolved in Tris buffer, and 0.1 ml. streptokinase (1000 units per ml.). The system was clotted and observed for lysis as described in the one-stage assay above. Concentration of E-ACA in the clots ranged from 6.25×10^{-6} M to 1.25×10^{-2} M. The total amount of activator developed in the clot was 0.07 unit in the case of canine plasma and 0.15 unit in the system containing human plasma. The amount of bovine profibrinolysin in the system was constant; 0.013 Guest unit (15) being supplied by the bovine fibrinogen and thrombin. The results are depicted in Table 5.

Discussion

The two-stage assay procedure measures the change in the rate of fibrinolysin generation induced by the concentrations of E-ACA present in the incubation mixtures (Tables 1 to 3). The effect of E-ACA on the second stage of the assay was reduced by a dilution of 4 to 10 fold before testing. Preliminary experiments with fibrinolysin generated under the conditions described indicated that losses in fibrinolytic activity which occurred during dialysis were often pronounced an invariably unpredictable. Thus dialysis of the samples to remove E-ACA prior to determination of fibrinolytic activity was not considered feasible.

TABLE 5

Effect of ε-aminocaproic acid on activity of streptokinase-elicited plasma activators

E-ACA concentration 0	Activator: source and concentration Canine 0.07*	Human 0.15*
	Per Cent Change from Control Values	
6.25×10^{-6} M	—37	— 6
1.25×10^{-5} M	—70	—36
6.25×10^{-5} M	—80	—50
1.25×10^{-4} M	—94	—66
6.25×10^{-4} M	—99	—90
1.25×10^{-3} M	—99	—96
6.25×10^{-3} M	—99	—99
1.25×10^{-2} M	—99	—99

* Activator concentration expressed in activator units (9) per 0.4 ml. clot.

The rate of development of canine fibrinolytic activity in the presence of staphylokinase exhibited a bazaar pattern in the presence of lower concentrations of E-ACA. At these lower concentrations, an actual increase in the rate of generation of fibrinolytic activity was observed. This may be a reflection of a phenomenon noted by Zylber *et al.* (33) with streptokinase-elicited human activator, bovine plasminogen, and pancreatic trypsin inhibitor. On the other hand it may simply be the result of enhanced stability of canine fibrinolysin in the presence of E-ACA. This point is currently under investigation. The latter conclusion appears more justified in view of the fact that when the incubation period was prolonged, the enhancement was more dramatic. At higher concentrations, the degree of enhancement became less and less marked until it was virtually absent at a concentration of E-ACA of 1×10^{-2} M. Inhibition of activator activity in the case of staphylokinase was not complete even at this concentration as indicated by pronounced activity present in aliquots withdrawn after prolonged incubation. Of interest in this connection is the observation by Norman (25) that certain amines tend to stabilize fibrinolysin.

At 50 minutes, the activator effect of urokinase upon bovine profibrinolysin was less inhibited than that of staphylokinase on canine profibrinolysin or urokinase on human profibrinolysin. These observations are not related to variations in sensitivity to E-ACA inhibition of the three fibrinolysins (see Table 4). While a difference in sensitivity of staphylokinase and urokinase to inhibition by E-ACA was not surprising, a difference between the effect of E-ACA on the rate of conversion of two different profibrinolysins to their respective fibrinolysins by human urokinase was unexpected. This is particularly true in view of the fact that the sensitivity to inhibition is greater in the case of the conversion of human profibrinolysin to fibrinolysin by the human urokinase. The reverse situation would be anticipated on the assumption that human profibrinolysin would constitute the best substrate for human urokinase.

The inhibitory actions of E-ACA are not restricted to agents capable of bringing about the conversion of profibrinolysin to fibrinolysin as indicated by the data of Table 4. Here as little as 6.25×10^{-4} M E-ACA produced a 40 to 70% inhibition of the various fibrinolysins tested. Twice this concentration of E-ACA

did not inhibit the action of either staphylokinase or urokinase. Concentrations of E-ACA which produced up to 90% inhibition of fibrinolysin brought about as little as a 41% inhibition of urokinase. It is apparent not only that E-ACA is inhibitory against fibrinolysin but also that concentrations of E-ACA which inhibit activators overlap those which inhibit fibrinolysin itself.

Results of the examinations of the inhibitory effect of E-ACA as determined in the one-stage systems containing streptokinase and human or canine plasma are more difficult to interpret because the fibrinolytic activity was measured in the presence of undiluted inhibitor. However, the data clearly indicate that the activator produced in human and canine plasma in the presence of streptokinase was more sensitive to inhibition by E-ACA than either urokinase, staphylokinase or the various fibrinolysins studied. It is not clear at this point whether E-ACA interferes with the action of the activator or its formation or both. Certainly it may be concluded with safety that concentrations of E-ACA which inhibited the streptokinase-elicited activator of canine or human plasma did not bring about appreciable inhibition of either canine or human fibrinolysin. (Compare Tables 4 and 5.)

The data of this paper do not justify the recent emphasis placed upon the primary significance of E-ACA as an inhibitor of agents bringing about the conversion of profibrinolysin to fibrinolysin (5, 6, 16, 24, 29, 31). Nor is this emphasis in harmony with the findings of Ablondi *et al.* (2, 3) and others (1, 26) all of whom have reported that E-ACA inhibits not only fibrinolytic activity but also trypsin. Our data leave no doubt that inhibition of fibrinolysin is possible at the same concentrations of E-ACA which are required for inhibition of activators.

The fact that E-ACA is an effective antifibrinolytic agent as well as a potent inhibitor of the activator generated in human plasma by streptokinase makes of it a valuable agent to have at hand during fibrinolytic therapy. This is particularly true when the therapeutic agent selected is streptokinase. Especially pertinent are the observations of Ablondi and De Renzo (2) that E-ACA does not inhibit activator and/or fibrinolysin adsorbed on a formed clot but does inhibit any activation processes going on in the fluid medium surrounding that clot.

It must be emphasized that this dual role of E-ACA as an inhibitor not only of activators but also of fibrinolysin, may

render it dangerous when used in excessive quantities. The physiologic role of the fibrinolytic enzyme system has yet to be evaluated. Much is known of the *in vivo* manifestations of an increase in the activity of the fibrinolytic system (27, 30). However, little is known about the consequences of its partial inhibition and nothing is known concerning the effects of its abolition.

The fibrinolytic enzyme system has been demonstrated in every species (7, 9, 18). The primitive nature of this system is indicated by the broad range of profibrinolysins which can be activated by human urokinase (23). For example, in this laboratory, it has been demonstrated repeatedly (9) that human urokinase can activate a proteolytic enzyme system in the blood of fish which is analogous to the urokinase activation of the profibrinolysins of man and other mammals.

Profound inhibition of not only the development of fibrinolytic activity but also that of existing fibrinolytic activity may have serious consequences. To date the dose regimen has been pushed as high as 36 gm./day by Nilsson and his colleagues (24). They report no untoward effects in short term studies. However, they point out that in cases where renal function is impaired, a considerable decrease in the rate of excretion of E-ACA may take place. Under such circumstances they believe that treatment with E-ACA is contraindicated.

The drug, E-ACA, because of limited availability and because of the rapidity with which it is excreted in patients with normal kidney function, has apparently not yet produced serious toxic manifestations. Nevertheless, we believe that E-ACA should be used with caution for the following reasons: (1) the inhibitor not only blocks the activation of profibrinolysin but also inactivates the fibrinolysin itself; (2) the consequences of inhibition of the fibrinolytic enzyme system over a period of time are not known; and (3) patients with impaired kidney function may develop high concentrations of the drug in the circulating blood. An examination of the literature reveals that E-ACA is rapidly growing in favor as an agent for the treatment of excessive bleeding in the presence or absence of excessive fibrinolytic activity, and in a variety of other conditions (10). Thus, a drug with a relatively restricted applicability is being used for many purposes; its value as a therapeutic agent could be nullified by ex-

cessive dosage and by use in conditions in which there is no basic or sound clinical rationale.

Summary

Data are presented which indicate that epsilon-aminocaproic acid (E-ACA) inhibits fibrinolysin as well as agents which bring about conversion of profibrinolysin to fibrinolysin. The concentrations of E-ACA required to inhibit such activator substances also inhibit fibrinolysin. The findings suggest that therapeutic use of large amounts of E-ACA should be undertaken with caution.

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SUPPRESSION BY CORTISONE OF LEUKERGY INDUCED BY ENDOTOXIN

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In spite of many investigations, the mechanism of the anti-inflammatory effect of corticosteroids and their effect on resistance are still far from clear. It is generally assumed that cortisone reduces resistance to infection, although it increases the resistance to endotoxin (1, 2, 3, 4, 8, 9). There are statements (23, 24) that ACTH and cortisone, like inflammation or responses to other stresses, enlarge the amount of alkaline phosphatase in the leukocytes of man. These last statements are rather surprising since both hormones are known as anti-inflammatory agents.

Among many other contradictory assumptions, there are also claims that the sticking of the leukocytes to endothelial cells of the small vessels as well as the phagocytosis by polynuclear leukocytes is reduced under the influence of cortisone (7, 10).

In view of this conflicting evidence we undertook to investigate the effect of cortisone on leukergy.

Leukergy (25–37) is a defense mechanism against infection: in certain states such as inflammation, some infections, pregnancy, large blood loss, there appear in circulating blood adhesive, particularly energetic leukocytes (*the leukergic leukocytes*). They have the tendency to marginate in the vessels and to become trapped in the inflammatory foci, by sticking there to the vascular endothelium. The leukergic leukocytes are especially rich in glycogen and alkaline phosphatase (32, 35, 37), they are more motile and phagocytize more than normal (29, 30). Animals in the state of leukergy are more resistant to infection (34). Owing to their adhesiveness, the leukergic leukocytes show *in vivo* and *in vitro* agglomeration with a marked cytological homogeneity of the clumps (Fig. 1). They can be identified and counted by means of a simple test (*the leukergy test*).

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Many authors—since the beginning of this century—have found adhesive leukocytes in exudates, and in blood during various diseases, in pregnancy, during menstruation, and in blood of animals after lipopolysaccharide injection (12, 18, 40, 41, 42, 44). Especially E. v. Philipsborn in Germany (1930) has done much work on increased stickiness of leukocytes and the connection with their motility and phagocytic activity. However, it was assumed that the already circulating leukocytes have undergone changes, and not that the hemopoietic organs produce a new type of adhesive leukocytes. No proper test for the differentiation of the adhesive leukocytes from the "ordinary" ones and no counting method for them has been described. Furthermore, the role of the adhesiveness in shifting of leukocytes to the infectious foci, as well as in their sticking to clumps of platelets in which bacteria are trapped, has not been recognized. The share of the lymphocytes in the phenomenon of sticking and agglomerating has not been mentioned.

The presence of an "iodophilic substance" or even of glycogen in certain leukocytes has been noticed already in the last decades of the 19th century (Ranvier 1877, Ehrlich 1883, Best 1903) (15, 19, 20, 21). This phenomenon was sometimes attributed to a degenerative process (15). Several authors mentioned also the presence or the increase of phosphatase in leukocytes of inflammatory and stress states (19, 20, 22, 38). Especially Valentine (23, 24) reported an increased amount of this enzyme in various states of stress.

An increase in the motility as well as in the phagocytic activity of "inflammatory leukocytes" has also been noticed (3, 18). Similarly, an increase of resistance to infection after injection of endotoxin (lipopolysaccharide) is widely known (3, 11, 51).

However, no relationship has been sought between the various phenomena reported.

The concept of leukergy offers a synthesis of this complex of various, independent findings and provides the phenomena concerned with a functional meaning.

The specific terms "leukergic leukocyte" and "aleukergic leukocyte" have been proposed. The name "inflammatory leukocyte" is misleading: there is no inflammatory state in normal pregnancy or following an intensive muscular activity or after profuse bleeding; nevertheless, many leukergic leukocytes are

present in these states. The term "stress leukocyte" would be similarly improper, since many stresses due to various intoxications or some virus infections do not evoke leukergy. Again, "normal leukocytes" are not always aleukergic: in pregnancy, in newborn infants there are perfectly normal leukocytes in blood, 30–40% of which are leukergic; and aleukergic leukocytes are not always normal, e.g. the leukocytes in leukemia are aleukergic and abnormal.

The leukergic leukocyte can be recognized, counted and traced in the bone marrow, in the systemic or pulmonary blood, in exudates and in pus.

Leukergy should be regarded as a special cellular defense mechanism which acts very promptly not only in various actual infections but also in some conditions of imminent danger of infection (wound with a great loss of blood, fatigue, shock, pregnancy). It is a very watchful mechanism. The finding that leukergy appears after spinal puncture or after lumbar air insufflation (49), as well as in some diseases of the diencephalon, suggests that this phenomenon may be controlled by a center in this region.

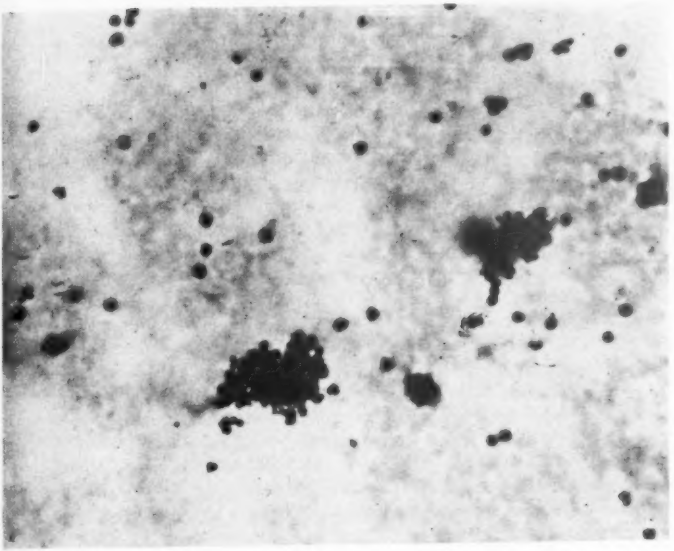
The appearance of leukergy in states preceding infection recalls the emergency theory of adrenal function (Cannon, 45). Fright alone, i.e. an imminent danger, is sufficient to evoke (by liberation of adrenaline into the blood stream) the dilatation of the vessels of the skeletal muscles, hyperglycemia, increased oxygen capacity of the blood, bronchiolar dilatation, shortened coagulation time of the blood, etc.—all reactions which prepare the animal for defense or flight (46).

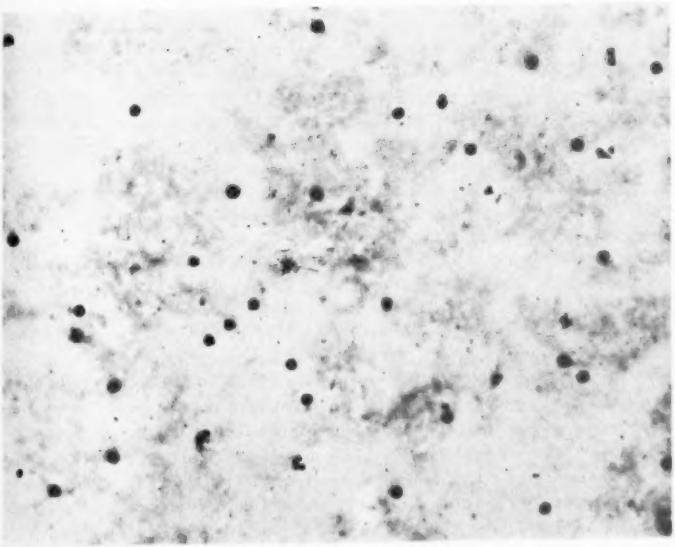
There arises a question: what is the controlling agent of the leukergic mechanism? Is it a hormone?

FIG. 1. The leukergy test in the blood of patient No. 11 (Table 3) with proliferations of the lymphatic glands mainly in the abdomen and the supraclavicular region. Total leukocyte count: appr. 7000. Blood picture: 60% lymphocytes, many of which contain glycogen granula.

a) Before treatment: 62% of the leukocytes clump together. In this picture three great clumps of granulocytes (of approx. 11,20 and 30 cells, resp.) and three small clumps of lymphocytes (3, 4 and 4 cells) can be seen.

b) After 3 days of treatment with Meticorten (50 mg daily): only 3% of the leukocytes clump together. In this picture no clumps visible.





Materials and Methods

Rabbits were used as experimental animals. Observations on human patients were also made.

Twelve adult (2500–3000 g) and 3 young (600–730 g) rabbits were injected intramuscularly with 50 mg/kg of Cortisone acetate (Upjohn) or Cortadren (Schering) every day for 3 days, whereas 15 other adult rabbits served as controls (without cortisone). On the fourth day all 30 rabbits received intravenously endotoxin from *E. coli* or *Proteus* × 19, prepared according to the method of Boivin-Mesrobeanu (47); the dose of our preparation was 0.5 mg/kg in saline. Immediately before injection, as well as five times afterwards (1 hour, 4–5 hours, 5–6 hours, 24 hours and 72 hours after the injection) the following tests were made: body temperature, leukocytosis, blood picture, leukergy test, glycogen and alkaline phosphatase in leukocytes. During these three post-injection days of observation the test animals continued to receive 50 mg/kg of the hormone. The course of the leukergic reaction to endotoxin injection has been described in previous papers (33, 35, 37).

Two other adult rabbits, treated for 3 days with cortisone as above, were given intravenously 2 ml/kg of peritoneal exudate of a healthy rabbit (prepared after de Haan). For comparison two other rabbits were injected with the same exudate without the cortisone pre-treatment. The course of the leukergic reaction to exudate injection has been described previously (27).

Some additional rabbits were used for the study of the effects of prolonged treatment with cortisone.

The *glycogen content* of the leucocytes was determined by the use of a semi-quantitative cytochemical method. One hundred granulocytes were counted in blood smears, stained according to Hotchkiss (48), and scored in five classes, from "negative" to "4 plus", depending on the intensity of staining. The sum of 3-plus and 4-plus cells, in relation to the total number of granulocytes counted, i.e. the percentage of strongly positive granulocytes (the glycogen-rich leukocytes) was used as an index of the increase of the glycogen cells. The percentages have been converted to absolute numbers/mm^3, according to the blood pictures and the total leukocyte counts/mm^3.

The *alkaline phosphatase* content was estimated in a similar way, using the modified Gomori method of staining (52).

The *leukergy test* is performed as follows: a citrated sample of blood (1 part of a 3.8% solution of sodium citrate and 4 parts of blood) is incubated at 37° C. for three hours. After gentle stirring, a drop is placed on a slide, rocked gently, dried in the incubator (37° C.) and stained with methylene blue without fixation. The percentage of agglomerated white cells on the slide is determined by microscopic observation, by counting 500–1000 cells. Cells are considered agglomerated when 3 or more of them are seen associated in a distance smaller than one diameter of the cell. From the percentage a absolute number of agglomerated cells/mm^3 was calculated according to the blood picture and the total leukocyte count/mm^3.

Mainly granulocytes agglomerate, only seldom lymphocytes (children, diseases with lymphocytosis). There is a marked tendency for cytological homogeneity of the clumps (Fig. 1) in the leukergy test preparations, i.e.

pure granulocytic or pure lymphocytic clumps are more frequent than would be expected statistically.

In addition to the experiments with rabbits, observations were made on 14 human patients. These patients, exhibiting mainly lymphatic proliferations, were treated with Meticorten and the leukergy test was performed on their bloods.

Care must be taken that all injections are performed with pyrogen-free water (saline). It is suspected that some contradictory statements in the literature were caused by the use of not pyrogen-free solutions. Fractions of one gamma of pyrogen are sufficient to provoke leukergy, i.e. an increase of glycogen and phosphatase content and a positive leukergy test.

Results

1. The results of experiments performed with 30 rabbits injected with endotoxin are summarized in Table 1. The values given in this table are means of individual values.

It was found that the elevation of the temperature was lower in the cortisone group than in the controls. The leukopenia in the first phase after injection of endotoxin did not reach the same degree as in control animals and lasted a shorter time.

During the 4–5th hour after injection of endotoxin the numbers of agglomerated cells and of glycogen rich cells were greater in cortisone treated animals than in controls.

One hour later, i.e. in the 5–6th hour after injection of endotoxin, the situation changes: the number of agglomerated cells is already greater in normal animals, and that of glycogen-rich cells of these animals becomes almost equal to the corresponding figure for cortisone treated animals.

Later on, 24 and 72 hours after injection of endotoxin, the preponderance of leukergy in normal animals is very clear. After 72 hours the nontreated animals show still a marked leukergic reaction, the cortisone treated ones practically none.

Table 1 contains no values for alkaline phosphatase, because of the great individual differences for the leukocytes of apparently healthy animals: there are healthy rabbits with 40% strongly positive granulocytes and others with only 15%. The percentage of positive granulocytes presents inexplicable short lasting fluctuations. However, a significant decrease of the phosphatase reaction was visible in all cortisone treated animals: e.g. the percentage of negative granulocytes increased gradually during a 5-day treatment (50 mg/kg daily) from 36% to 92%, or (in

TABLE 1

Average values in 15 normal and 15 cortisone treated rabbits

	Before injection of endotoxin					After injection of endotoxin (0.5 mg/kg)																							
						1 hour					4–5 hours					5–6 hours					24 hours					72 hours				
	T	L	Gr	A	Gl	T	L	Gr	A	Gl	T	L	Gr	A	Gl	T	L	Gr	A	Gl	T	L	Gr	A	Gl	T	L	Gr	A	Gl
Normal	39.1	8121	3800	162 (2%)	0	39.5	1600	640	0	51 (8%)	40.2	4690	2300	500 (10.5%)	1300 (57.7%)	41.1	8975	6000	3300 (37%)	4000 (68.5%)	39.7	25800	20500	16400 (65%)	1600 (8%)	39	9500	5200	3300 (35%)	0
Cortisone treated	38.8	5822	4350	87 (1.5%)	0	39.1	2300	1290	0	26 (2%)	39.9	10700	8500	2300 (22%)	4600 (54%)	39.7	10100	9000	1700 (16.6%)	5000 (55%)	39.7	21200	19000	8800 (42.2%)	380 (2%)	38.8	6800	5400	340 (5%)	0

Cortisone given 50 mg/kg daily for 3 days before endotoxin and 3 days after endotoxin.

T—body temperature
L—leukocytosis/mm³
Gr—granulocytes/mm³
%—per cent
A—number of agglomerated cells in the leukergy test/mm³ (in brackets : expressed as % of the white cells).
Gl—number of granulocytes with a strong glycogen staining/mm³ (in brackets : expressed as % of the granulocytes).

another case) from 50% to 62%. These animals showed after endotoxin injection an increase in strongly positive cells, but this increase became smaller after a longer cortisone treatment.

Changes of the blood picture were seen in all cortisone treated animals: a marked decrease in the lymphocyte count and a small increase in the granulocytes.

All cortisone treated rabbits lost about 10–15% of their weight during the experiment. All of them showed a marked muscle-weakness. Two young animals (700 and 730 g) and one adult died, and post mortem examination of them revealed bronchopneumonia (1) and enteritis (2), respectively.

Ten of the cortisone treated rabbits were sacrificed after close of the observation. No gross pathological changes were found. There was no cortical necrosis of the kidneys.

2. Three adult rabbits were treated with cortisone (25–50 mg/kg daily) for 2–5 weeks. They lost about 20–25% in weight during this time. An intravenous injection of endotoxin into these animals was repeated three times during the course of the cortisone treatment and the response was observed. Previous experiments (50) showed that the presence of specific antibodies against the endotoxin did not influence the leukergic response.

Also the development of a tolerance, which takes place after several injections of pyrogens repeated in short intervals, does not repress the leukergic agglomeration even when fever and leukocytosis are no longer present (50). It is permissible therefore, to repeat for our purposes the injection three times at intervals of several days.

Table 2 depicts the reaction of rabbit No. 43 treated with cortisone for two weeks. The changes of the reaction to endotoxin are here even more distinct than in Table 1: a shorter leukopenic phase, and an earlier appearance, in systemic blood, of adhesive and glycogen-rich cells. The whole reaction lasted a shorter period of time: for 24 hours after the injection of endotoxin, the percentage of agglomerated cells as well as the percentage of glycogen-rich cells after the cortisone treatment were below the pretreatment level. The second rabbit reacted similarly. In the third rabbit (No. 25) the response to three times repeated injections of endotoxin during a five-week treatment with cortisone became gradually weaker. Finally there was no leukergic response at

TABLE 2

Reaction of rabbit No. 43 during a prolonged treatment with cortisone

		Before injection of endotoxin					After intravenous injection of endotoxin									
							4 hours					24 hours				
		T	L	Gr	A	Gl	T	L	Gr	A	Gl	T	L	Gr	A	Gl
Before cortisone treatment		39.3	11000	6000	0	0	41.3	2300	1200	0	672 (56%)	40.4	70000	63000	60000 (86%)	6300 (10%)
After cortisone given for:	3 days	38.9	6500	5700	0	0	39.3	19000	17900	11400 (60%)	10700 (60%)	39.3	27000	25400	17600 (65%)	1000 (4%)
	8 days	39.6	10300	8100	0	0	39.9	12000	11200	5200 (43%)	8500 (76%)	39.2	18000	16000	4500 (25%)	0
	14 days	39.2	9900	8000	0	0	39.4	24000	21600	11500 (48%)	19500 (90%)	39.2	30000	25500	2100 (7%)	0

Daily cortisone dose : 50 mg.
Explanations as in Table 1.

all. There was a rise in the number of circulating leukocytes (from 4000 to 13,000), but they were not leukergic.

At this stage, rabbit No. 25 was injected intrapleurally with 0.5 ml. of terebinth oil. On the following day the leukergy test remained negative, although in animals not treated with cortisone such an injection evokes the appearance of 40–70% of leukergic agglomeration. After withdrawal of the pleural exudate from the test rabbit, the rabbit was killed. The post mortem revealed a fully developed cortical necrosis of both kidneys. The pleural exudate was turbid, hemorrhagic, with few white cells, mainly mononuclears. When 3 ml of this exudate was injected intravenously into a healthy rabbit, no leukergy appeared in it. However, a moderate leukocytosis (rise from 9000 to 14,000) with shift to the left was provoked. An injection into a healthy rabbit of a similar exudate from a normal rabbit, caused a very quick appearance of leukocytosis (rise from 8000 to 15,000) with leukergy (47%).

In the exudate of the rabbit No. 25, described above, the factor provoking leukergy was apparently absent.

3. In the group of four rabbits which were given intravenously the peritoneal exudate from a healthy rabbit, there was no difference between the two animals pretreated with cortisone and the two controls: all of the rabbits developed, after 20–40 min., leukocytosis accompanied by increase in glycogen content and leukergic agglomeration. The reaction disappeared after approximately 10 hours.

4. Table 3 sums up the observations made on 14 human patients mainly with lymphatic proliferations, treated with *Meticorten* (50 mg daily for 7–10 days). Nine of them showed clinical improvement (reduction in the size of lmyphoid tissue), accompanied by a decrease of the leukergy test. In case No. 11 the effect was dramatic: after three days of treatment with 50 mg Meticorten daily, the percentage of adhesive cells fell from 62% to 3% (Fig. 1). In the remaining 5 cases neither a clinical improvement nor a decrease of leukergy were seen. It seems that the leukergy test can be helpful in tracing the therapeutic effect of hormones.

Comments

It has been established that the leukergic reaction proceeds after the injection of endotoxin differently in cortisone treated

animals and in untreated ones: in cortisone treated animals the leukopenic phase is less deep (Table 1, first hour) and shorter (Table 1, fourth hour). The mean number of leukocytes decreased in noncortisone treated animals from 8120 to 1600, i.e. by 80%, while in cortisone treated rabbits it did so from 5822 to 2300, i.e. by 60% only. At the end of the fourth hour the leukopenic phase is over in cortisone treated animals, while it is still marked in the normal ones.

If one compared the systemic blood in the two groups at the end of the fourth hour after injection of the endotoxin, one would conclude that the reaction of the cortisone treated rabbits is stronger than that of non-cortisone treated animals: at that time the cortisone rabbits show twice as many leukocytes as the non-cortisone treated ones, more than fourfold agglomeration and threefold the number of glycogen-rich cells (Table 1). One should however remember that at this time the composition of the systemic blood depends on two antagonistic processes:

1) the *leukopoiesis* manifested by the increase in the number of leukocytes, mainly of granulocytes with a shift to the left. The newly formed leukocytes can be leukergic (*leukergic leukopoiesis*) or not (*aleukergic leukopoiesis*): this can be established by their agglomerability in the leukergy test and by their relative phosphatase and glycogen content.

2) *the trapping of the leukocytes, mainly in the pulmonary circulation* (16, 17, 36). This occurs chiefly with the leukergic leukocytes, but also with the aleukergic ones. The significance of this phenomenon for the clearing of blood from microorganisms has been established (36). The trapping of leukocytes in the pulmonary circulation manifests itself by leukopenia (mainly granulocytopenia) in the greater circulation and by granulocytosis in the punctate of the lungs. Such a punctate contains during the pulmonary leukostasis a greater percentage of glycogen-rich cells than the systemic blood (36). If before the leukopenic phase the blood was leukergic, the percentage of leukergic cells in the systemic blood drops during the leukopenic phase (36), because mainly leukergic, glycogen-rich cells are shifted into the pulmonary circulation.

The relative strength of the two processes—*leukopoiesis* and *trapping*—determines the composition of the systemic blood. In the fourth hour after the application of endotoxin (in the dose

used), trapping still prevails in the normal animals, so that *leukopenia* is still pronounced.

The blood of the cortisone treated animals shows after injection of endotoxin distinct signs of leukopoiesis: in all cases, even after prolonged treatment with cortisone, there was a significant increase of the leukocyte count with the appearance of young forms of granulocytes. Nevertheless, the percentage of agglomerated leukocytes (in the leukergy test) was—with the exception of the fourth hour after injection of endotoxin—always lower than in normal controls: 16.6% against 37%; 42.2% against 63%; 5% against 35% (Table 1, similarly in Table 2). The same is true of the percentage of glycogen-rich cells: 55% against 68.3%; 2% against 8% (Table 1) and 4% against 10% (Table 2). After prolonged cortisone treatment we found only 7% and 0% of leukergic cells in spite of significant leukocytosis (30,000 or 13,000 resp.).

These results lead us to believe that in cortisone treated animals there was a leukopoiesis, but it gradually became aleukergic. There is no reason to assume that in the fourth hour a stronger leukergic leukopoiesis took place than later on, especially as after the first hour only 2% of glycogen-rich cells were found (Table 1). Therefore, it can be assumed that the stronger leukergy test with the systemic blood of cortisone treated animals four hours after endotoxin originates from a weaker trapping of leukocytes in the pulmonary circulation, not from a stronger leukergic leukopoiesis. The weaker leukopenia in peripheric blood points to weaker pulmonary trapping.

The trapping mechanism in the pulmonary vessels—the stickiness of the capillary walls—acts in normal conditions jointly with the leukergic stickiness of the leukocytes. This mechanism, which has an important defense function, viz. the shifting of the leukocytes to the place of their functional destination (36), is impaired by cortisone.

The cortisone treated animals show a weaker leukergic response, and it seems that in their exudates less of the intrinsic leukergy provoking agent is present. Further investigation in this direction is necessary.

No differences were found between cortisone treated animals and the controls in respect to the reaction to intravenous injection of exudates from normal animals. It is not impossible that

by such an injection the agent lacking in cortisone treated rabbits (the intrinsic leukergy provoking agent) is directly supplied.

Summary

1) The leukergic response to endotoxin in rabbits treated for three days with cortisone (50 mg/kg), as estimated cytochemically and by the leukergy test, is depressed and lasts shorter than in control animals.

2) During a prolonged treatment with cortisone the leukopoiesis which follows an endotoxin injection becomes gradually aleukergic.

3) The weaker leukopenia in the greater circulation of cortisone treated rabbits in the first phase after endotoxin injection, suggests that the mechanism of trapping leukocytes in the pulmonary circulation is impaired.

4) In a case of total loss of the leukergic reactivity after a prolonged treatment with cortisone, there was no leukergy provoking agent in the pleural exudate elicited by terebinth oil, con-

TABLE 3

Leukergy test in 14 patients with lymphatic proliferations, before and after treatment with Meticorten

Diagnosis	Treatment*	Leukergy before treatment (per cent)	Leukergy after treatment (per cent)	Effect of treatment
(1) Chronic Lymphatic Leukemia	Meticorten alone	52	No change	No change
(2) idem	Meticorten alone	38	No change	No change
(3) Reticulo Cell Sarcoma	Meticorten alone	25	7	Better
(4) Hodgkins Disease	Meticorten alone	32	No change	No change
(5) idem	Meticorten alone	23	17	Bit better
(6) idem	Meticorten alone	21	11	Bit better
(7) Acute Leukemia	Meticorten alone	23	9	Better
(8) Plasmocytoma	Meticorten alone	27	45	Worse
(9) Carcinoma of ovary	Meticorten + Antibiotics	38	5	Better
(10) Breast Carcinoma	Meticorten alone	32	No change	No change
(11) Lymphadenopathia	Meticorten alone	62	3	Much better
(12) Lymphosarcoma	Meticorten alone	35	12	Better
(13) Reticulo Cell Sarcoma	Meticorten alone	30	15	Better
(14) Hodgkins Disease	Meticorten alone	32	10	Better

* 50 mg Meticorten daily for 7–10 days.

trary to such exudates originating from normal animals. The exudate of the cortisone treated animal provoked an aleukergic leukopoiesis.

5) The changes in the reaction of cortisone treated rabbits point to an impairment of the important antibacterial defense mechanism provided by leukergy.

6) No differences were found between cortisone treated animals and the controls in respect to the reaction to intravenous injection of exudates from normal animals. It is postulated that by such an injection the agent lacking in cortisone rabbits (the intrinsic leukergy provoking agent) is supplied.

7) Out of 14 human patients suffering mainly of lymphatic proliferations, nine showed a clinical improvement (reduction in the size of lymphoid tissue) after a short treatment with Meticorten. In these cases a marked decrease of the leukergy was noticed. In the remaining five patients neither clinical improvement nor a decrease of leukergy was observed. The leukergy test may be useful in assessing the therapeutic effect of anti-inflammatory hormones.

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THE RELATIONSHIP BETWEEN WECHSLER-BELLEVUE DIGIT SYMBOL PERFORMANCE AND PSYCHOSIS

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The psychological literature is replete with studies concerned with the diagnostic efficiency of the Wechsler-Bellevue Intelligence Scale (W-B). Many attempts have been made to determine the relationship between various diagnostic classifications and W-B subtest patterns. For the most part the results of such studies have been inconclusive and, certainly, little in the way of consistent findings has emerged. Guertin, *et al.* (1956), as a result of their surveys of this literature, were prompted to conclude that little could be gained by the continuation of such an approach.

While these results have been disappointing, to some extent this may be because of the manner in which the problem of pattern analysis has typically been approached. In most of those studies, individuals were first grouped according to diagnosis, and then the W-B patterns were examined for possible clusters which were homogeneous within each of the several diagnostic groupings. An alternative approach is to select a group of individuals on the basis of test patterns and then to examine this group for diagnostic similarity. Furthermore, it was felt that the clinical observations, involving the Digit Symbol subtest, on which this study was based, were sufficiently consistent to justify investigation.

The Digit Symbol test is generally considered a measure of new learning efficiency involving visual-motor coordination. While used for many years as a measure of intelligence, it has been relatively neglected regarding its possible implications for differential diagnosis of psychopathology. Wechsler (1944) probably gave less attention to the Digit Symbol test than any of the other subtests in his remarks about clinical applications of the W-B, although he did imply that both schizophrenics and neu-

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[b] University of Houston. Paper accepted for publication Nov. 10, 1960.
[c] In an unpublished study based on over one thousand patients referred for psychological testing here at the Medical Branch, Roessle reports a sex ratio of 52% female and 48% male.

rotics tend to have low scores on this test. Only Rappaport (1946) has explored the diagnostic possibilities of the Digit Symbol test in detail. He contended that low Digit Symbol performance is generally indicative of a pathological depression or schizophrenic disturbance, but that some bland schizophrenics, as well as preschizophrenics, may obtain high weighted scores. However, Rappaport concluded that: ". . . comparison of the Digit Symbol score with the other Performance subtest scores taken together is relatively ineffective for diagnostic purposes."

The clinical staff at the University of Texas Medical Branch had noted some similarities among patients who obtained a relatively high score on the Digit Symbol subtest. Most of these patients evidenced behavior that was often classified as psychotic. It was further observed that most of these patients were female. It was the purpose of this study to investigate these two clinical observations.

Procedure

The records of all psychiatric patients hospitalized at the University of Texas Medical Branch during the preceding year, and who were given the routine battery of psychological tests, were obtained. Form I of the Wechsler-Bellevue Intelligence Scale was included in this battery of tests and was administered in the usual manner, except that the Object Assembly subtest was omitted. These records were examined for age, sex, W-B IQ, and psychiatric diagnosis. Patients who obtained a full scale IQ below 70 and those diagnosed as having organic brain pathology were excluded from the study; no further limitations were imposed. Since both private and charity patients were included, representing a wide range of socio-economic levels, it was felt that these patients were a representative sample of hospitalized psychiatric patients in this area. An experimental group and a control group were selected from these 576 cases. The criterion for the experimental group was simply that the Digit Symbol subtest was the highest of the Performance subtests by at least one weighted score point (DS High). A total of 41 such cases were found. The control group was obtained by selecting, for each of the 41 DS High cases, the next name in sequence in the files which was of the same sex. Thus, the two groups were matched for sex, but for none of the other variables. These two groups were then compared with respect to the incidence of psychoses. The relationhsip between sex and high Digit Symbol performance was also examined.

Results

Table 1 shows that the two groups did not differ significantly on either age or IQ.

Psychosis. Table 2 indicates the frequency of psychosis in each of the two groups, and substantiates the prediction of a greater incidence of psychosis in the DS High group.

Sex. Of the total 41 DS High patients, 33 (80.49%) were female and 8 (19.51%) were male. A Chi-Square comparison of this proportion against an expectation of 52% female and 48% male yielded a value of 14.06 (df = 1), which was significant beyond the .001 level. Thus, both predictions were verified at a high level of statistical significance.

Discussion of Results

The finding that high Digit Symbol performance is significantly related to the incidence of psychosis does not in any way indicate that psychotic patients will necessarily show this pattern of the W-B. It does, however, suggest that when this pattern occurs, there is a high probability that some psychotic involvement is present in the patient. Actually, this pattern is not a particularly common one, but when it is found it should alert the clinician to the possibility of a psychotic disorder.

Although it has been demonstrated that there are no significant sex differences with respect to general performance on the Digit

TABLE 1

Descriptive data for the two groups

	DS high group	Control group	Significance level
Age	Mean 26.37	Mean 31.93	t .362
	σ 10.33	σ 11.37	Not Sig.
IQ	Mean 98.83	Mean 105.71	t .350
	σ 12.30	σ 14.64	Not Sig.

TABLE 2

Frequency of psychosis within each group

	Psychotic	Non psychotic	Significance level
DS high group	33	8	$\chi^2 = 18.00$
Control group	14	27	$< .001$

Symbol subtest of Form I of the Wechsler-Bellevue (Goolishian & Foster, 1954), it is interesting to note that the DS High pattern occurred significantly more often among females.

The diagnostic criterion of a psychotic-nonpsychotic dichotomy used in this study is admittedly very gross. Although the study was not concerned with the ocurrence of different psychotic conditions within this broad diagnostic classification, a *post hoc* consideration of this point revealed some interesting trends. It was noted that the DS High patients often presented a diagnostic problem. Most of them evidenced a definite psychotic quality to their thinking but without the loss of affect often associated with schizophrenics. Further, in four of eight nonpsychotic cases in the DS High group, the possibility of psychosis was considered at some time during their hospitalization. Also, many of these patients seemed to achieve an apparent remission of their psychotic symptoms in a relatively short period of time. Although generalizations are not possible, these patients appear to be similar in many respects to Rappaport's preschizophrenics referred to earlier. In any event, DS High patients appear to share some common behavioral characteristics. Further research is underway to explore systematically the characteristics of this group.

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FOOD INTAKE STUDIES IN GOLDTHIOGLUCOSE OBESE CBA MICE[a]

GEORGE F. GRAY[b] AND ROBERT A. LIEBELT[c]

The placement of electrolytic lesions in the ventro-medial region of the hypothalamus in various animal species results in a hyperphagia that is followed by a massive and persistent weight gain (3, 4, 8, 9, 15). The induced hyperphagia is only transient. The experimental rats return to pre-operative food intake levels after a period of time with persistence of the obesity (4, 9). This pattern of increased food intake is repeated when the animals are allowed free access to food following a period of starvation. However, certain animals with hypothalamic lesions will continue to over eat and to gain weight almost indefinitely (4, 20).

In mice, a single injection of goldthioglucose results in the development of lesions in the ventro-medial area of the hypothalamus (7, 12, 17) as well as other areas of the brain (12). These animals subsequently develop a permanent obesity (2, 6, 14). Waxler and Brecher (21), who originally described the development of obesity in goldthioguclose-treated mice, reported a persistent hyperphagia for a period of twenty weeks in injected stock ablino mice. The experiments were carried out with animals which had received an LD 50 dose of goldthioglucose, and the extent of hypothalamic damage in each instance was unknown. More recently, it has been found that mice of the CBA strain show a uniform weight gain with negligible mortality following the intraperitoneal injection of 0.4 mg. per gram of body weight of goldthioglucose (12, 13). These animals likewise show a relatively uniform lesion size in the hypothalamus following this dose of goldthioglucose. It was the purpose of this investigation to study the food intake of goldthioglucose-treated CBA mice: 1) during the immediate post-injection period when allowed to feed *ad libitum*, 2) following a restriction of food during the immedi-

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ate post-injection period, and 3) following a period of starvation at 4 months post-injection.

Methods and Materials

Male and female mice of the CBA strain were used. This strain has been maintained in this laboratory for over twenty generations by brother-sister mating. The animals were housed individually in 6″ × 12″ × 6″ wooden cages with a ¼″ wire mesh false bottom. Each cage was furnished with a metal food hopper which permitted free access to powdered Purina lab chow. Food intake and body weight were measured at daily intervals in short term studies, and at three day intervals in the experiments of longer duration.

1. *Food intake during the immediate post-injection period.* Twenty mice were placed in individual cages at approximately 90 days of age. Body weight and food consumption were recorded for a 30 day control period. At 120 days of age all animals were injected with 0.4 mg. per gram of body weight of goldthioglucose (Solganol B[d]) intraperitoneally. Food intake and weight gain were followed for a period of 60 days post-injection.

2. *Food intake following dietary restriction for 30 days post-injection.* Ten animals were allowed a 30 day period for adaptation, and then injected with 0.4 mg. per gram body weight of goldthioglucose at 120 days of age. The animals were given a four gram pellet of Purina lab chow each day (it had been previously determined that the daily averaged food consumption during the 30 day control period was approximately four grams). This restricted food intake was continued for 30 days post-injection, and then the animals were allowed free access to powdered chow from the food hopper. Body weight and food intake were recorded for an additional 30 days.

3. *Food intake of obese mice allowed to feed* ad libitum *after starvation.* Fourteen obese and eight control CBA mice of a similar age (7 months) were used in this study. The obese animals had received a single injection of goldthioglucose approximately four months earlier. The animals were allowed a 30 day adaptation period, and then all food was removed. Water was continually available. Obese animals were starved for an average of ten days or until a 40 to 50% weight loss was recorded. The animals were then allowed to feed *ad libitum*. The eight control animals were subjected to the same procedure. However, these animals were permitted to lose only 20 to 30% of body weight before being returned to free access to food.

Results

Food intake was markedly decreased for one to two days immediately following the administration of goldthioglucose, but increased to almost twice the pretreatment levels within the next 8–10 days (Fig. 1). Food consumption then gradually returned to control levels by 28–30 days post-injection. Food intake remained at this level for the remaining 30 days of this study

[d] Courtesy of the Schering Corporation, Bloomfield, New Jersey.

(Table 1). The period of greatest weight gain corresponded to the time of greatest food intake. As food consumption returned to control values, the rate of weight gain correspondingly decreased. However, the animals remained at the increased body weights (fourteen to eighteen grams above control weights). No animal was observed to lose weight as the rate of food intake decreased.

When the food intake was restricted (a potential maximum of 4 grams per day) for 30 days after goldthioglucose administration, there was no significant increase in body weight (Fig. 2). However, when the animals were permitted to feed *ad libitum*

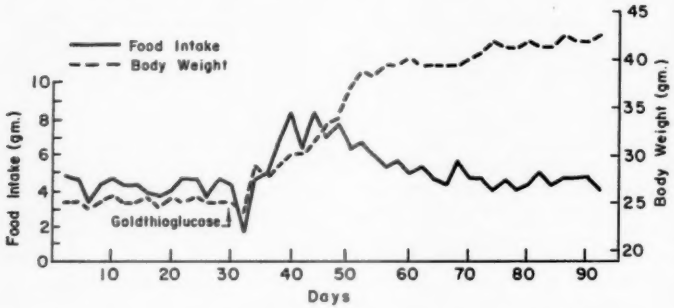

FIG. 1. Relationship between food intake and body weight following a single injection of goldthioglucose in a CBA mouse.

TABLE 1

Food intake of CBA mice injected with Goldthioglucose

Animal number	Total food intake (GMS)		
	Control period (30 days)	Post-injection period	
		Days 1–30	Days 31–60
1	126.3	153.4	131.2
2	132.0	159.3	129.7
3	111.4	135.8	120.4
4	114.2	156.0	123.6
5	120.7	141.5	117.3
6	135.6	156.8	129.3
7	99.3	135.2	117.8
8	117.0	158.3	122.1
9	129.6	157.4	126.8
10	114.3	152.0	125.3

after this period there was a pattern of food intake and weight gain very similar to that seen in animals allowed free access to food immediately after goldthiogulcose administration (Table 2).

Mice which had been obese for approximately four months demonstrated a hyperphagia when they were fed *ad libitum* after starvation (Fig. 3). The hyperphagia was accompanied by a very rapid return to pre-starvation weight. The period of increased food intake was not as long in duration as that seen immediately following the administration of goldthiogulcose, nor was the

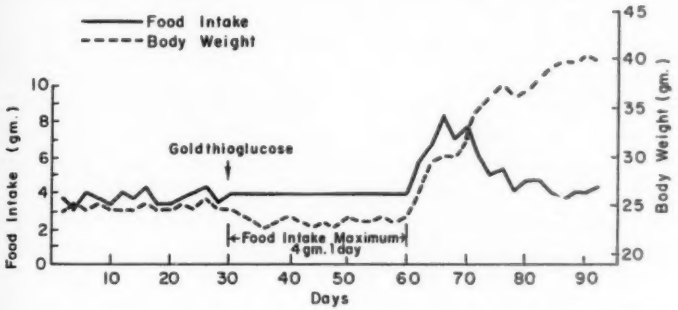

FIG. 2. Relationship between food intake and body weight after a restricted food intake for 30 days post-goldthioglucose injection.

TABLE 2

Food intake of Goldthioglucose treated CBA mice on restricted food intake during the immediate post-injection period

Animal number	Total food intake (GMS)		
	Control period (30 days)	Post-injection period	
		Days 1–30	Days 31–60
1	127.6		163.6
2	118.4		152.0
3	116.8		154.6
4	103.0		141.3
5	121.5	Potential	149.8
6	110.6	Maximum	138.6
7	109.3	of 120 gms	146.3
8	129.2	per mouse	152.3
9	114.6		158.3
10	118.9		161.2

amount consumed as constant in magnitude. Control mice of the same age as the obese animals exhibited similar periods of hyperphagia following starvation, but to a lesser degree. Animals from the control group did not tolerate starvation to the same degree as obese animals and three died within a week or less after food deprivation. On the other hand, the obese animals tolerated 10 days or more of starvation before becoming moribund. Animals from both groups lost weight rapidly when deprived of food. Resumption of feeding *ad libitum* resulted in a rapid return to pre-starvation weights accompanied by a transient hyperphagia (Fig. 3).

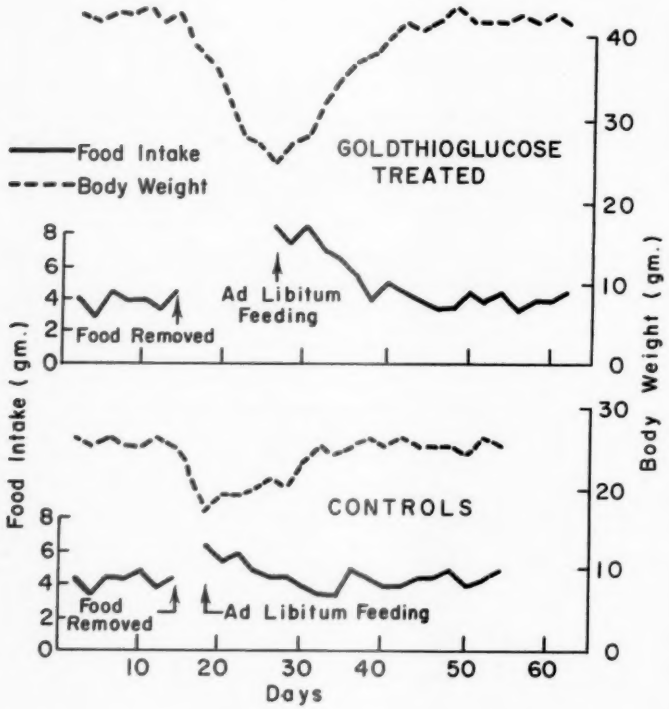

FIG. 3. Food intake pattern of obese and control CBA mice placed on *ad libitum* feeding after a period of starvation.

Discussion

The physiological determinants of appetite control are considered to be divided into two main areas: central or primary regulation and peripheral or secondary regulation. Central regulation is considered to be directed by hypothalamic centers, but the exact nature of this regulation is incompletely understood. Brobeck and his associates (5) have related the control of food intake to temperature regulation of the body. Mayer (16) proposes that the hypothalamus has elements which are sensitive to altered arterio-venous glucose differences, and Kennedy (10) has offered evidence to support the idea that levels of circulating metabolites whether derived from digested food or from the turnover of the fat depots control appetite in intact animals. Anand and Brobeck (1) have demonstrated that different hypothalamic areas may have varied functions in regard to food intake: destruction of the lateral hypothalamic areas causes anorexia in contrast to the hyperphagia seen following ventro-medial lesions. The effect of ventro-medial lesions, of whatever etiology, is apparently to destroy, at least partially, the central "satiety" apparatus. The nature of secondary appetite regulation is not well understood. The capacity of the stomach, the contractions of the empty stomach, and palatability of the available food supply may be involved (11). The importance of the latter is most strongly suggested by the poor adaptation to dietary dilution seen in animals with either electrolytically or goldthioglucose induced hypothalamic lesions (4, 11, 18, 19, 20).

The results of the present experiment are similar to the findings obtained in rats with electrolytically induced lesions; that is, the hyperphagia seen in goldthioglucose-treated CBA mice is not permanent, but is temporally related to the period of rapid weight gain which is primarily lipid deposition. The return to pre-treatment food intake levels in goldthioglucose induced obesity is not a function of a compensatory reorganization in the damaged hypothalamic region, but rather is a permanent alteration in appetite regulation. Mice on restricted food intake during the period immediately following goldthioglucose treatment demonstrate hyperphagia at a time when treated mice fed *ad libitum* have returned to normal food intake level. Also obese mice will show the same pattern of hyperphagia when starved and then permitted to feed *ad libitum* as long as four months after receiving

goldthioglucose. Of significance is the similarity in response between obese and control mice to starvation followed by free access to food. Both groups of animals showed a transient hyperphagia lasting until the starvation-induced weight loss was restored. These findings suggest that goldthioglucose-induced hypothalamic destruction alters only a particular component of the appetite regulating complex rather than inducing a generalized disturbance. Furthermore, the secondary controls on appetite regulation appear to consistently maintain the organism at the new body weight after destruction of the primary regulatory center.

The basis for the difference between the persistent hyperphagia reported by Waxler and Brecher (21) in goldthioglucose-treated albino mice as compared to the transient hyperphagia oberved in CBA mice in the present study remains to be determined. These data need not be interpreted as conflicting since both types of feeding responses have been reported in rats with hypothalamic lesions.

Genetic factors have been shown in various strains of mice to influence the response to goldthioglucose in terms of weight gain and toxicity (13). Likewise, a relationship has been noted between the amount of goldthioglucose injected and the subsequent number of grams increase in body weight. Unpublished observations suggest a correlation between the extent of hypothalamic damage and the amount gained in body weight. The different patterns of hyperphagia observed in goldthioglucose-treated albino mice and CBA mice could be explained on the basis of differences in the size of the induced hypothalamic lesions. The transient hyperphagia and obesity seen in CBA mice receiving relatively small doses of goldthioglucose may be due to partial destruction of "satiety" centers, whereas persistent hyperphagia and obesity would result from larger doses. The possible quantitative relationship between the degree of destruction of the hypothalamic "satiety" centers and food intake warrants further investigation.

Summary

The food intake of goldthioglucose treated CBA mice was studied 1) during the immediate post-injection period, 2) following dietary restriction for 30 days post-injection, and 3) in obese mice 4 months post-injection. Injected mice showed a transient hyper-

phagia lasting approximately thirty days and then returning to control levels. A similar food intake pattern was observed in mice which were restricted to a maximum of 4 grams per day during the first 30 days post-injection and then fed *ad libitum*. Obese mice were starved for various periods of time and then fed *ad libitum*. These animals were hyperphagic until the starvation-induced weight loss was restored and then returned to control levels of food intake.

These data demonstrated that the weight gain observed in CBA mice following a single injection of goldthioglucose was associated with a transient rather than a persistent hyperphagia, and this unique alteration in appetite regulation was permanent.

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UROKINASE: PHYSIOLOGIC ACTIVATOR OF PROFIBRINOLYSIN[a]

M. Mason Guest and D. R. Celander[b]

The primary object of this presentation is to summarize some of our physiologic observations relative to urokinase made since its discovery in 1951–52 (3, 24, 26). For a number of years our efforts consisted chiefly of an essentially chemically oriented inquiry into conditions most adequately suited to the preparation and quantitative estimation of urokinase (5, 7, 8, 9, 17, 20) together with the development of methods which we believed to provide reliable information regarding alterations in the plasma fibrinolytic enzyme system (2, 6, 13, 14, 15). More recently we have undertaken a physiologic study of the various changes produced by urokinase when administered to animals and in the variations of urokinase excretion as influenced by a variety of circumstances.

Preparation and Assay of Urokinase

In Fig. 1 is depicted in schematic form the method which we currently employ in the preparation of urokinase in quantities sufficient to permit at least limited physiologic inquiry. The key features consist in the concentration of urokinase in urine foam, partial purification by chromatography on columns of commercially available silicic acid (21) and finally a purification regimen which involves chromatography on DEAE-cellulose. The validity of our one stage assay for urokinase (Fig. 2) with respect to measuring the kinetic activity of urine has been established by a variety of experimental procedures (5, 7, 9). The

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assay procedure involves the lysis of a profibrinolysin-containing fibrin clot formed in the presence of the urokinase to be measured. The dependence of the assay procedure upon the presence in the system of profibrinolysin is illustrated in Fig. 3. Equivalent concentrations of urokinase were added to a relatively profibrinolysin-free human fibrinogen preparation, clotted with profibrinolysin-free thrombin, and to a conventional preparation of bovine fibrinogen clotted with Parke, Davis Topical Thrombin. There is almost no relationship between the amount of urokinase added (abscissa) and the amount of urokinase measured (ordinate) in a system poor in profibrinolysin whereas there is an obvious, direct relationship between the kinase added and kinase measured in our conventional assay system. This evidence indicates that the assay procedure measures urokinase.

The centiunits depicted on the abscissa of the assay curve (Fig. 2) are roughly equal to those employed by Smyrniotis *et al.* (23) in their publication dealing with variations in urokinase excretion in man. The unit (4) which is comprised of 100 of these centiunits, is approximately 10 times the unit described by Ploug and Kjeldgaard (22) in 1957 and about 1/10 of the unit employed by Von Kaulla in certain of his published findings (25).

Excretion of Urokinase

Using this assay procedure, 78% of the presumed normal individuals whose urine we have examined exhibit a 24-hour excretion of urokinase ranging from approximately 300 to 900 units. Removal or augmentation of salt intake while it produces dramatic changes in urokinase excretion does not result in values lying outside this range. Individuals on a low salt diet appear in the 900 unit category, while those consuming diets high in salt are in the approximately 300 unit range. Since all samples are dialyzed against phosphate buffer prior to analysis, these findings cannot be related to chloride inhibition of urokinase (10). The maximum extremes in urokinase excretion which we have encountered in presumed normal individuals have been from as low as 200 units to almost 1200 units per 24 hours. In a single individual who was working daily and was apparently normal, the 24-hour urokinase excretion varied from 243 to 1180 units over a period of 9 days.

In patients suffering from neoplastic disease, urokinase excre-

tion tends to be somewhat depressed, 33% of the patients exhibiting a 24-hour urokinase excretion of less than 300 units. A tendency for a depressed urokinase excretion has also been observed in approximately 50% of patients suffering from muscular dystrophy. In this group the 24-hour excretion values range between 150 and 300. The amount of urokinase excreted by patients treated with intramuscular trypsin did not materially deviate from the amount excreted by untreated, presumably normal individuals. Excretion of urokinase in a limited number of cardiac patients (approximately 30 days post infarct) was found to be markedly depressed. One patient in this group excreted only 45 units in 24 hours. In patients with severe third degree burns a decrease in excretion of urokinase tended to occur. An extreme case of this type was an 89-year old female who had suffered first, second and third degree burns over 88% of her body. This patient excreted in 24 hours a total of 3 units of urokinase.

The data on urokinase excretion were derived from 131 individuals, 67 of whom were presumed to be normal. The total number of determinations performed was approximately 300. The patient groups examined ranged in size from 4 to 20. These data are not definitive; as presented, they constitute only a guide for future investigation. Furthermore, two problems arise in the determination of urokinase by all methods which we have so far tested or developed: (1) the possible presence of inhibitors to the enzyme which are not removed by the dialysis step which precedes our assay (8); and (2) the possibility that more than one urokinase may be present in human urine. In the experiment depicted in Fig. 4, partially purified urokinase was placed on a column of diethylaminoethyl (DEAE)-cellulose. During elution at constant ionic strength, pH, and temperature, two distinct kinase peaks separated by a number of inactive fractions were obtained. Differences in the behavior of the slope dilution curves in these two samples of urokinase were observed. This means that an assay procedure calibrated to measure an average mixture of urokinases will likely provide erroneous data if applied to a urine sample in which either one or the other of these urokinase species predominates. We are continuing our assays of urokinase excretion but emphasize that excretion data unless obtained under well defined and highly controlled conditions may be misleading.

In the final analysis a genuine evaluation of the significance of

excretion studies depends upon whether or not urokinase originates systemically or is solely an extravascular product of kidney activity. Data in this area are confusing. Kjeldgaard has remarked (16) that intravascular administration of human urokinase to man was not followed by its enhanced excretion in urine. Data obtained by Von Kaulla (25), Smyrniotis (23) and in this laboratory regarding the variation in urokinase excretion can hardly be accounted for on the basis of its being produced solely by extravascular kidney tissue. On the other hand Ladehoff (18) has shown that the superficial tissue of the renal pelvis and ureters are rich in activator; he attempts to associate the activator in urine with the activity, turnover, and sloughing of these tissues. Certain data of our own presented in detail elsewhere (7, 9) indicate that in heterologous studies, human urokinase injected into rabbits gives the following results: (1) the amount of urokinase excreted in rabbit urine is sharply increased; (2) this urokinase can be isolated by procedures which were designed for the isolation of human urokinase and which are not ordinarily practicable for the isolation of rabbit urokinase; and (3) the urokinase isolated from the urine of injected rabbits will induce anaphylactic death in guinea pigs sensitized to human urokinase. It is important to emphasize that while these latter findings indicate that human urokinase can cross the rabbit glomerulus, they do not constitute definitive evidence that urokinase is produced systemically.

Urinary Proactivator

Nearly all urine samples which we have examined exhibit an augmentation of activator activity when treated with exogenous streptokinase. Thus the increase in apparent urokinase activity, reported in myocardial patients treated with preparations containing streptokinase (23), may actually reflect activation by streptokinase of proactivator normally present in the urine. The presence of streptokinase-activatable material in human urine which exists simultaneously with active urokinase suggests to us that this streptokinase-sensitive material is not profibrinolysin since any profibrinolysin present could have been expected to have been activated by the urokinase present. The findings just presented appear to provide evidence for the presence of a proactivator independent of profibrinolysin.

The proactivator in urine is increased under controlled physiologic conditions. With exercise at approximately 75% of capacity (i.e. 75% of that required to produce a heart rate of 180, and R.Q. above 1, as established by R. T. Clark and associates while at the School of Aviation Medicine, Randolph Field), streptokinase-elicitable activator rises abruptly in the urine and there is concurrently a decrease in plasma proactivator which, in some individuals in the older age groups, amounts to as much as 50%. Concomitantly there develops only a mild lytic activity as measured by lysis times of clots formed from carbon dioxide precipitated plasma globulin fractions (11) in spite of the fact that there is a material augmentation of circulating activator as determined by the cold fibrinolysis technique (14).

in Vivo Effects of Urokinase in Dogs

The findings which we have obtained from our studies in man have been extended by studies in animals where more rigorous, experimental procedures may be employed. Our first preparations of soluble urokinase were obtained in February, March and April of 1953. This grossly impure material was injected intravenously into two dogs. Both animals responded within minutes by an elevation in body temperature and an acute toxic reaction culminating in a shock-like state. Both animals subsequently recovered without supportive therapy. However, one of them died later of a bacteremia which we believe was unrelated to the urokinase injection. The other animal not only survived the experiment but bore several litters of normal puppies over the two year period during which she was kept for observation. Repeated administration of the same crude preparation available at that time induced only a mild, transient febrile response (1° to 2° F.) in rabbits. No grossly discernible ill effects were observed in the treated rabbits.

In 1957, we injected intravenously into dogs our first colorless, relatively purified urokinase preparations in what we believed to be massive doses. In a typical experiment a 12 kg. dog, anesthetized with Na pentobarbital (3 mg/kg), was infused intravenously over a period of 10 minutes with 6000 Celander and Guest units (8) (60,000 Ploug and Kjeldgaard units (22)) of urokinase in 29 ml of 0.05 M phosphate buffer, pH 6.8.

Within the first 90 minutes there was an abrupt, nearly 30%

increase in the cold fibrinolytic activity (reflecting we believe in part circulating activator) which fell to 20% below pre-injection values at the end of 24 hours. Thirty minutes after the infusion, the fibrinogen decreased by 22%, remained stationary for the next hour and then rose to a level of approximately 50% above pre-injection values by the end of 24 hours. The fibrinogen rise may be related to stress. (Dr. J. M. McKenzie, working in our laboratory, has shown (19) that, in the dog, response to even mild, non-traumatic stress is uniformly reflected by elevations in the fibrinogen titer which require as much as 45 days of tranquillity to abolish.) The profibrinolysin titer fell rapidly. By the end of 90 minutes it had been reduced to 60% of pre-injection values. It continued to decrease until by the end of 24 hours it had reached a value less than half of that observed prior to the urokinase infusion. The decline in proactivator was almost as rapid as that observed for profibrinolysin. However, at the end of 24 hours, circulating proactivator concentration had risen to a point which was 9% in excess of the preinjection value. Antifibrinolysin also declined at first. By the end of 90 minutes it had decreased by 12% and this decrease continued, although more slowly until at the end of 24 hours it had reached a level 20% below preinjection values. We have since injected dogs with doses of urokinase ranging from 168 to 1705 units per kg and have obtained results essentially similar to those just described. However, a good correlation between the magnitude of the dose of urokinase employed and the degree of the change in the injected animals was not obtained.

In 1956, Dr. S. N. Kolmen, who was then associated with us in research, observed that erythrocytes possessed a high degree of affinity for a variety of factors associated with the fibrinolytic enzyme system. Of particular interest was his observation that urokinase was readily adsorbed from solution *in vitro* by thoroughly washed erythrocytes of several species (17). Following adsorption of urokinase, these erythrocyte preparations exhibited activator activity; i.e. when placed in the presence of profibrinolysin, they readily brought about its conversion to fibrinolysin. Dr. Kolmen's *in vitro* findings stimulated interest on the part of Dr. H. J. Stuart in the possible *in vivo* application of the adsorption phenomenon. This interest was engendered by the fact that the urokinase adsorbed to erythrocytes not only retained its ac-

tivity but operated more effectively in the presence of inhibitors than did free urokinase. In order to enhance the adsorption of the protein, urokinase, to erythrocytes Stuart thoroughly washed the red cells and then exposed them to 1:20,000 tannic acid (4) in the regimen depicted in Fig. 5. He was able to achieve a considerably enhanced adsorption of active urokinase on the "tanned" canine erythrocytes. He then injected into dog veins free urokinase, tanned erythrocytes which had not been exposed to urokinase, and tanned erythrocytes carrying a maximal load of urokinase. A comparison of urokinase administered directly compared with urokinase administered after adsorption to washed, tanned red cells is presented in Fig. 6. The urokinase adsorbed to washed, and tanned erythrocytes produced a somewhat different pattern (Fig. 6B) in the response of the factors measured than did urokinase alone (Fig. 6A). Subsequent investigation of the effects produced by tanned erythrocytes alone (Fig. 6C) gave results which may provide valuable information regarding the role of surface active agents in producing alterations in the fibrinolytic enzyme system. It may be noted in Fig. 6B that when tanned erythrocytes which had been exposed to urokinase were injected intravascularly in a typical experiment, alterations in the fibrinolytic system, resembling those previously described as well as those in Fig. 6A, occurred. The one noteworthy exception is that antifibrinolysin which declined rapidly in the presence of free urokinase failed to decrease markedly until 3 days following the infusion of the urokinase-adsorbed tanned erythrocytes. An examination of the data in Fig. 6C reveals a surprising effect, namely that tanned erythrocytes alone were able to induce changes in various components of the fibrinolytic enzyme system. There was a marked fluctuation in the titer of fibrinogen in excess of what constitutes normal variation and an increase in antifibrinolysin. The mechanism through which these changes were brought about is not clear; possibly some factor which normally complexes with antifibrinolysin (1) was removed from it by the tanned erythrocytes. There was a sharp decrease in profibrinolysin concentration which did not return to values equal or above normal for seven days. While this change was not as great as that observed with any of the animals treated with urokinase, free or adsorbed, its occurrence is of considerable interest. The greatest change occurred in circulating proactivator

which fell to approximately 50% of pre-injection values and by the end of the experiment (that is 7 days) was still 25% below pre-injection levels. Again the question may be asked, was the proactivator adsorbed by the erythrocytes or was it utilized in the conversion of profibrinolysin to fibrinolysin through some sequence of reactions taking place on the surface of the treated erythrocytes. Data in Fig. 7 indicate that when tanned erythrocytes were introduced into the circulation of a normal dog a steady increase in fibrinolytic activity took place until the end of the first day. This enhanced fibrinolytic activity lasted at least 7 days. Experiments are now under way to ascertain just how long it can be expected to continue.

These experiments with a surface active agent capable of selectively adsorbing components of the fibrinolytic enzyme system and apparently permitting their reaction in an environment relatively unaffected by inhibitors is of interest to us and we sincerely hope that others will undertake experiments with similar agents with the view of achieving a new method in the control of the fibrinolytic enzyme system. Certainly today there is no agency available which will produce a sustained enhancement of fibrinolytic enzyme activity. Washed, or washed and tanned, erythrocytes may provide a device by which sustained fibrinolytic activity can be induced little by little by their gradual administration. Careful but not yet complete examination of the dogs which have been subjected to the parenteral administration of relatively large amounts of tanned erythrocytes with or without adsorbed urokinase has revealed no deleterious effects. Some of the dogs so treated have remained alive and well in our laboratory for a period of up to two years. Furthermore, the preparations were administered to trained animals without anesthesia and they exhibited no outward manifestations of discomfort. Fever, nausea, vomiting, diarrhea, excessive micturition and other typical stress reactions were entirely absent.

Summary

Urokinase excretion data are reported for presumed normal individuals, for patients with neoplastic disease, muscular dystrophy, cardiac disease, severe burns, and for individuals treated with intramuscular trypsin. Normal range is 300 to 900 units per 24 hour period. Currently available assays for urokinase are

limited by the possible presence, in varying amounts, of inhibitors to the enzyme as well as by the possibility that there may be more than one molecular species of urokinase.

Activator activity of most urine samples examined is augmented by addition of exogenous streptokinase. This proactivator activity is increased in the urine of individuals following exercise at approximately 75% of capacity. Examination of blood samples from such individuals reveals a concurrent decrease in plasma proactivator, a moderate increase in plasma lytic activity (as measured by euglobulin lysis time), and a material augmentation of plasma fibrinolytic potential.

Intravenous administration of urokinase to dogs, either free in solution or adsorbed to the surface of "tanned" red cells, brought about changes in components of the plasma fibrinolytic enzyme system indicative of activation of this system. In an extension of these studies, it was found that the intravenous administration of "tanned", homologous red cells alone without adsorbed urokinase was also effective in bringing about an apparent activation of the canine fibrinolytic enzyme system. The possible significance of surface active agents, such as "tanned" red cells, in the *in vivo* control of the fibrinolytic enzyme system is discussed.

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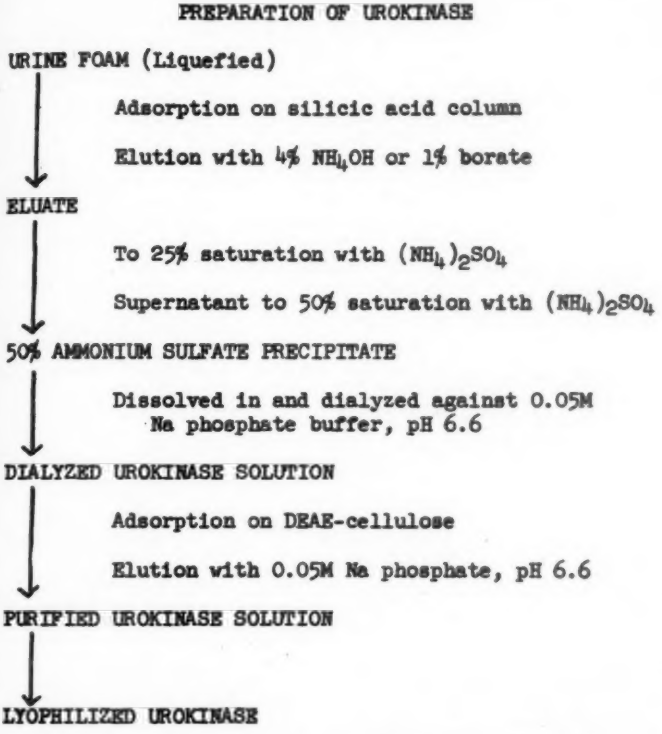
PREPARATION OF UROKINASE
URINE FOAM (Liquefied)
Adsorption on silicic acid column
Elution with 4% NH_4OH or 1% borate
ELUATE
To 25% saturation with $(NH_4)_2SO_4$
Supernatant to 50% saturation with $(NH_4)_2SO_4$
50% AMMONIUM SULFATE PRECIPITATE
Dissolved in and dialyzed against 0.05M Na phosphate buffer, pH 6.6
DIALYZED UROKINASE SOLUTION
Adsorption on DEAE-cellulose
Elution with 0.05M Na phosphate, pH 6.6
PURIFIED UROKINASE SOLUTION
LYOPHILIZED UROKINASE

FIG. 1. *Preparation of Urokinase.* Foam derived from whole urine by shaking a container, filled ⅓ full of pooled human urine, through 200 oscillations at a speed of 2 to 4 oscillations per second. Liquid layer siphoned off at end of 15 minutes and discarded. Remaining foam broken by a drop or less of capryl alcohol. Prior to placement on silicic acid column, material precipitable at pH 9 removed. Silicic acid column, prepared from 10% suspension AR silicic acid in 2M NaCl, contains 1 gram silicic acid per 2000 units urokinase to be adsorbed. Column is washed with 20 volumes distilled water prior to elution with ammonium hydroxide.

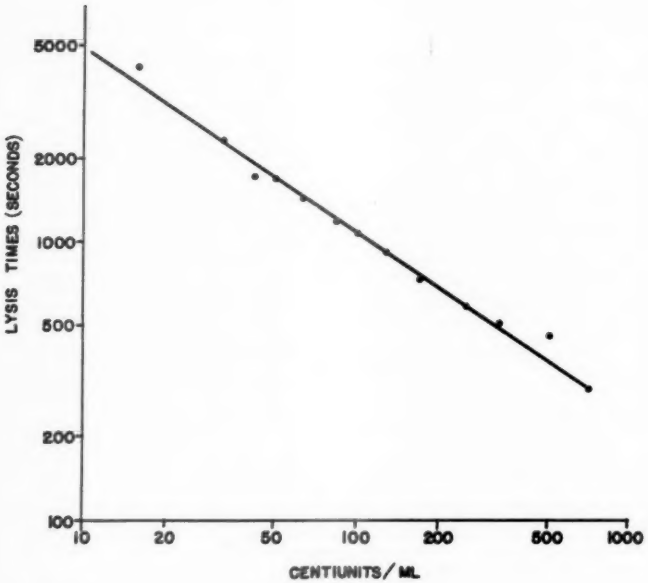

FIG. 2. *One Stage Assay Curve for Urokinase.* Reaction mixture: 0.2 ml 0.2% bovine freeze-thaw fibrinogen, 0.2 ml whole urine (previously dialyzed against 50 volumes of M/15 phosphate buffer, pH 7.25, 20 hours) or purified urokinase preparations made up in the same buffer or buffer of equivalent ionic strength and pH. Clotted with 0.005 ml bovine thrombin (Parke-Davis, 1400 units/ml). Lysis times determined by the tilt-tube method (26) at 37° C. and converted to centiunits/ml of solution assayed by reference to dilution curve for 0.4 ml system.

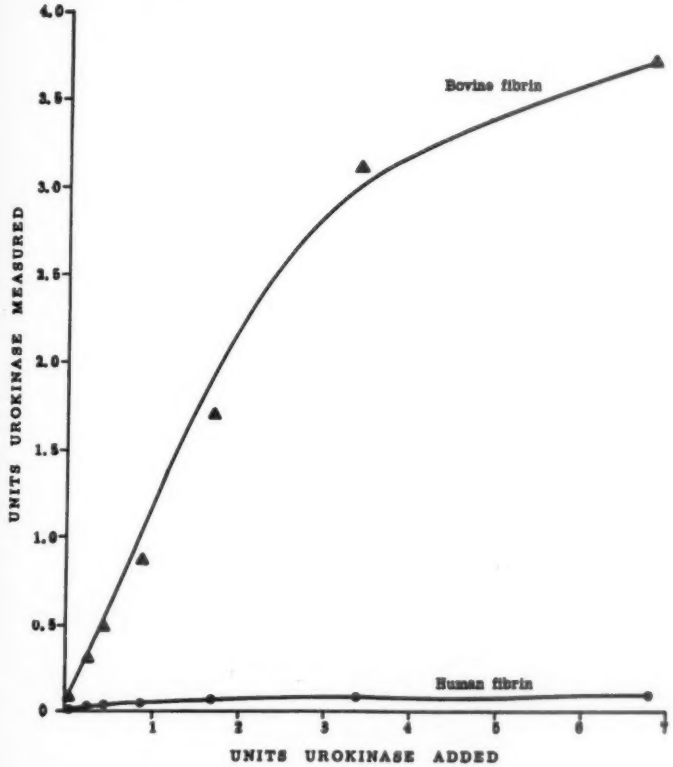

FIG. 3. *Sensitivity to Urokinase of Clots Prepared from Bovine Fibrinogen and Human Fibrinogen.* Bovine system: 0.2 ml bovine fibrinogen, 0.2%; 0.2 ml urokinase dilution; 0.005 ml Parke-Davis thrombin (280 units/ml).

Human system: 0.2 ml human freeze-thaw fibrinogen, 0.2%; 0.2 ml urokinase dilution; 0.005 ml Seegers' thrombin (200 units/ml).

Lysis times observed at 37° C., converted to units urokinase from the one stage assay curve (Fig. 2) and plotted against units of urokinase known to have been added.

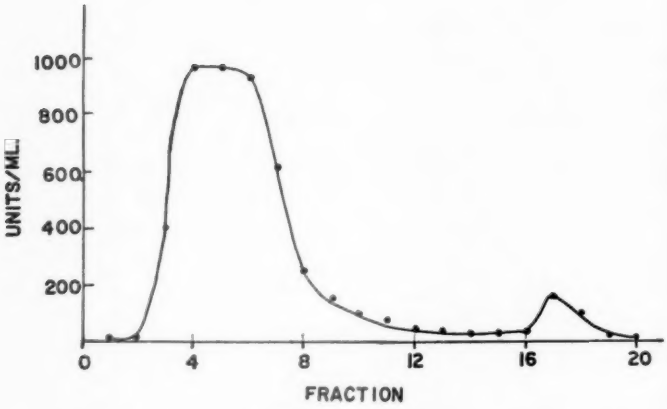

FIG. 4. *Fractionation of Urokinase on DEAE-Cellulose.* Activity of dialyzed urokinase solution (see Fig. 1) is determined and a column containing approximately 1 mg. DEAE-cellulose per 100 units urokinase to be chromatographed is equilibrated with 0.05 M phosphate buffer, pH 6.6. Urokinase solution is carefully poured on to column, followed immediately by five 20-ml. batches of the same buffer. Eluate collected in 5 ml. fractions which were diluted and assayed for urokinase activity by the one stage method (see Fig. 2).

PREPARATION OF UROKINASE-ADSORBED, "TANNED" CANINE RED CELLS
WHOLE DOG BLOOD (40 ml., 1:8 citrate)
Centrifuge, 20 min., 1500 x g
PACKED RED CELLS (15 ml.) + plasma
3 saline-phosphate buffer washes, pH 7.2
Centrifugation and dilution with buffer, pH 7.2
4% PACKED RED CELLS (1 volume)
+
1:20,000 TANNIC ACID (1 volume in saline)
Incubation at 37° C., 10 min.
Centrifugation, wash with pH 7.2 buffer
Redilution with pH 7.2 buffer
"TANNED" 4% RED CELL SOLUTION (1 volume)
+
UROKINASE SOLUTION (1 volume)
(5.64 units/ml., in buffered saline, pH 6.4)
Incubation at 37° C., 15 min.
Centrifugation, wash with pH 7.2 buffer
UROKINASE-ADSORBED, "TANNED" RED CELLS
(Resuspended in saline-phosphate buffer, pH 7.2, 20 ml.
injected into original donor dog)
Adsorption Efficiency = 56% (range 20 - 70%)

FIG. 5. *Preparation of Urokinase-Adsorbed, Tanned Red Cells.* The final volume in milliliters of washed, packed red cells is considered to represent 100% red cells and is diluted with 24 volumes of buffer to give a final concentration of 4% red cells. Although considerable amounts of residual urokinase were found in the supernatant of urokinase-adsorbed, tanned red cells, urokinase activity was not found in the first wash of such cells. Tanned, red cell preparations, not exposed to urokinase, were washed once with pH 7.2 buffer after incubation with 1:20,000 tannic acid, resuspended in a volume of the same buffer approximately equal to 50% of the whole blood withdrawn, and reinjected in the donor animals.

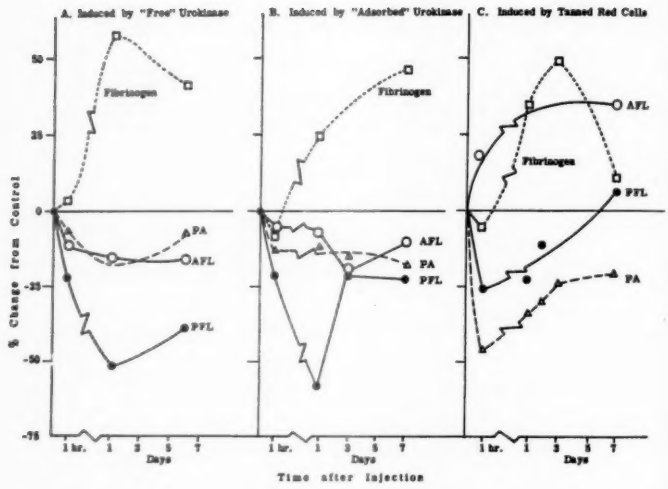

FIG. 6. *In Vivo Changes in Components of Canine Fibrinolytic System.* A. Induced by the intravascular administration of 1705 units/kg of urokinase dissolved in saline. B. Induced by the intravascular administration of 6.6 units/kg of urokinase adsorbed on surface of tanned erythrocytes (see Fig. 5). C. Induced by the intravascular administration of tanned erythrocytes not exposed to urokinase (see Fig. 5). Abbreviations used: PA, proactivator; PFL, profibrinolysin; AFL, antifibrinolysin.

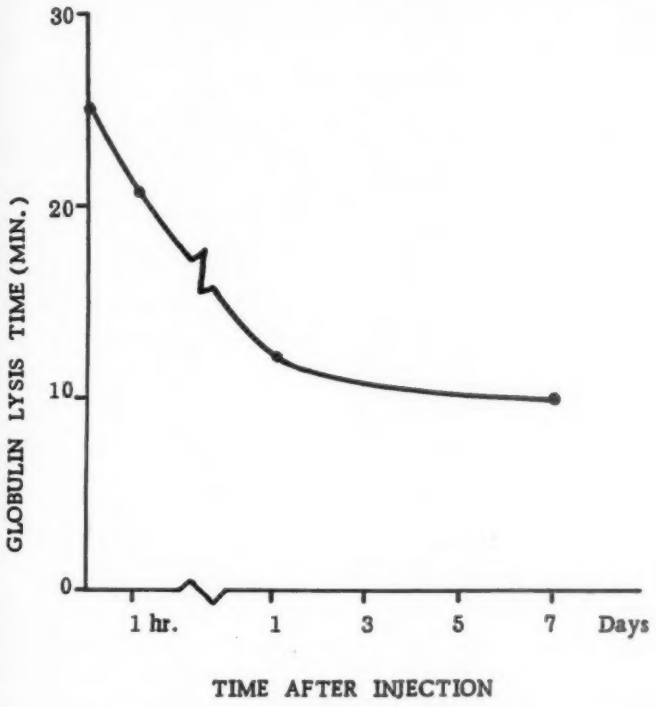

FIG. 7. *Lytic Activity Induced in vivo by Administration of Tanned Red Cells.* Tanned, red cells, prepared as described in Fig. 5, were injected into an unanesthetized dog. Plasma from samples drawn before injection of cells, and at 60 minutes, 24 hours, and 7 days after injection, were checked for lytic activity by a modified euglobulin lytic method. Euglobulins precipitated by carbon dioxide saturation of diluted plasma were collected by centrifugation and diluted to the original plasma volume with 0.9% saline. Equal volumes of such solutions were mixed with equal volumes of bovine fibrinogen (0.2%), clotted with thrombin, and observed for lysis at 37° C.

CYTOCHEMICAL AND FLUORESCENT ANTIBODY STUDIES ON THE GROWTH OF POLIOVIRUS IN TISSUE CULTURE[a]

HEATHER DONALD MAYOR[b]

Two extremely versatile techniques have in the past few years added much to our knowledge of the intracellular localization of viruses and their cycles of development: one is the fluorescent antibody method of Coons and Kaplan (11) which locates specifically viral antigens, the other the acridine orange technique developed independently by Armstrong (3) and von Bertalanffy and Bickis (6) which can be used as a cytochemical technique for demonstrating intracellular nucleic acids. A number of investigators have used one or the other of these techniques in studies of host-virus relationships.

Influenza (36), the g-antigen and the hemagglutinin of fowl plague (7), poliomyelitis (8, 9, 18), primary atypical pneumonia (20) and polyoma (14, 22) are but some of the viral antigens which have been studied using the fluorescent antibody technique. Influenza (2) and West Nile (2) are RNA viruses whose intracellular growth has been studied with the acridine orange technique. Tenenbaum (34) has used acriflavin, a fluorochrome inferior to acridine orange as a cytochemical stain to follow the distribution of nucleic acids in monkey kidney cells infected with poliovirus. Adenovirus (4), herpes simplex (31), vaccinia (5) and psittacosis (33) are among the DNA viruses which have been investigated. However, only in the report on herpes virus (31) was an integrated study including both fluorescent antibody methods for detecting viral antigen and fluorochrome techniques for viral nucleic acids presented.

Recently interest has been focused on the effect of vital, photodynamically active dyes (of which acridine orange is an example) on the infectivity of virus particles. The action on both mature and developing particles has been studied. LoGrippo and

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Basinski (21) have shown that the whole poliomyelitis particle is not inactivated by the action of acridine orange and ultraviolet light in contrast to the T3 bacteriophage. Hiatt (15) has demonstrated that the enteroviruses are unaffected by exposure to toluidine blue and light while herpes B, vaccinia, rabies, simian vacuolating viruses, and to a lesser extent adenovirus and reovirus are inactivated. Crowther and Melnick (12) reported that while mature poliovirus incubated with neutral red and exposed to white light showed no reduction in titer, virus grown in cells containing the dye could then be inactivated, indicating incorporation of the dye into the virus particle. Schaffer (32) obtained direct evidence for the incorporation of proflavin into poliovirus during its growth in tissue culture and assumed that the dye becomes bound to the RNA of the virus particle. The experiments reported here represent an attempt to present an integrated cytochemical and biological study on the developing poliovirus particle in tissue culture. Studies with labelled antibody, acridine orange staining of fixed tissue culture preparations before and after digestion with nucleases to establish specificity of nucleic acid identification, and experiments with the fluorochrome in living, infected cells are recorded and correlated with biological titrations of the infected material.

Materials and Methods

Tissue Culture. Monolayer cultures of trypsinized rhesus monkey kidney epithelial cells were grown on 11 × 22 mm coverslips in Leighton tubes using methods and Melnick's lactalbumin hydrolysate medium previously described (25).

Virus. The following virulent strains were studied: Mahoney (type 1), MEF_1 (type 2), Saukett (type 3). The following attenuated strains were studied: LSc (type 1), Y-SK (type 2), Leon (type 3).

Growth of Virus at 37° C. The maintenance medium was removed and the monolayers washed in the Leighton tubes three times with Hanks' balanced salt solution. The cultures were inoculated with 0.5 ml of a virus suspension containing approximately 10^7 PFU/ml. As there were approximately 10^6 cells in the monolayer this corresponded to an approximate multiplicity of 5 in each case.

The virus was allowed to adsorb for 1 hour at 37° C., the cultures were then washed 5 times with 5 ml of warm Hanks' solution to remove unadsorbed virus. Because of the stimulatory effect of cysteine on poliovirus yields, 1 ml of the cysteine medium described by Melnick, *et al.* (26) was then added to each tube and incubation continued at 37° C. Infected cultures were harvested every 2 or 4 hours for 24 hours after inoculation, and also examined for

cytopathic effect (C.P.E.). At each harvest time 2 cultures were kept for titration, 4 for fluorescent antibody, and 6 for cytochemistry including nuclease digestion tests. Control cultures in cysteine medium were processed at the same time and in the same manner.

Growth of Virus at 30° C. Monolayer cultures were processed in an identical manner to those at 37° C. except that after an initial adsorption time of 1 hour at 37° C. and a subsequent 2 hour incubation time in cysteine medium at 37° C. they were then placed at 30° C. and harvested at 2 or 4 hourly intervals for approximately 24 hours (30). Control cultures were treated in the same way. After microscopic examination for C.P.E. cultures were harvested for titration, fluorescent antibody and cytochemistry.

Growth of Virus in Presence of Acridine Orange. Infected cultures were inoculated with virus as previously described and allowed to adsorb for 1 hour. After thorough washing 1 ml of cysteine medium containing 1/200,000 acridine orange was added to each culture. This dilution of the dye was found in introductory experiments to be most satisfactory for preserving viability of the cells and for observation in the fluorescence microscope. The tubes were immediately protected from photodynamic inactivation by ordinary light (12) by covering each rack of tubes closely with aluminum foil. They were then incubated either for approximately 21 more hours at 37° C., or after 2 additional hours at 37° C., placed at 30° C. until 21 hours had elapsed

Titration of Intracellular Virus. For all experiments at 37° C. and 30° C. 2 tubes were processed at each time interval for titration of intracellular virus. The supernatant fluids (extracellular virus) were discarded and the coverslips washed 3 times while still in the Leighton tubes with warm Hanks' solution. 1 ml of fresh Hanks' solution was added to each tube and the cultures frozen flat so that the coverslips were completely covered with medium at —20° C. and stored until titrated for intracellular virus (26) by the plaque assay method of Hsiung and Melnick (17). For cultures grown up in presence of acridine orange titrations were carried out solely after 22 hours incubation, as in vital staining experiments; interest was focused mainly on cells known to contain a high titer of intracellular virus.

Fluorescent Antibody Staining Technique. The following sources of antibody were used: A pool of type 2 and 3 monkey antisera (neutralization titer 1/2500 against type 2, 1/6300 against type 3 as titrated by the panel test (27)), a type 1 antiserum (neutralization titer 1/12,500).

Globulin fractions of pooled sera were precipitated with half saturated ammonium sulphate at 0° C. and conjugated with fluorescein isothiocyanate by the established techniques (11, 29, 23) at a concentration of 10 mgm nitrogen/ml. Conjugates were passed down a column of anion exchange resin (Dowex 2 chloride*) to remove unconjugated isothiocyanate and stored in 5 ml volumes at —20° C. Before use each conjugate was adsorbed twice with acetone extracted monkey liver powder to remove nonspecific fluorescence, clarified at 18,000 rpm for 20 minutes in the Spinco preparatory centrifuge and filtered through a gradocol membrane pore size 0.45μ. The direct staining technique was used.

* Dow Chemical Company.

Coverslips were removed from tubes using a long fine wire hook, immediately dried in air for 30 minutes and fixed in acetone for 10 minutes. After a further brief drying period they were covered with 2 or 3 drops of neat conjugate, incubated at 37° C. in a humid chamber for 30 minutes, washed for 10 minutes in 3 changes of phosphate buffered saline pH 7.2 and mounted on slides in glycerol containing 10% buffered saline. Control preparations included uninfected cultures and infected cultures which had been previously incubated for 20 minutes at 37° C. with unlabelled antisera and washed for 10 minutes in buffered saline before testing. Both these preparations gave no specific fluorescence except a faint autofluorescence when stained with the anti-viral conjugate.

Cytochemical Staining Technique. Coverslips were removed as described above and placed immediately in Carnoy's fluid for brief fixation, usually 5 minutes at room temperature. They were then transferred directly to absolute alcohol, hydrated in ethyl alcohol series and after 5–10 minutes in McIlvaine's citric acid—disodium phosphate buffer at pH 4.0, stained for 5–10 minutes in 0.01% acridine orange solution in McIlvaine's buffer with initial agitation. After rinsing for 2–3 minutes in fresh McIlvaine's the coverslips were blotted gently and mounted on standard microscope slides using McIlvaine's buffer at pH 4.0 as mounting medium.

Vital Staining Technique. Monolayer cultures were harvested as described and placed in McIlvaine's for a few minutes to bring the pH to 4.0. Some coverslips were then mounted in buffer without additional staining and examined in the fluorescence microscope. Others were examined after Carnoy or alcoholic fixation both before and after subsequent staining with additional acridine orange according to the standard procedure.

Nuclease Digestion Tests for RNA and DNA.

RNA. Carnoy fixed coverslips were incubated in 0.05% 5 × crystallized ribonuclease[1] at pH 4 in McIlvaine's buffer as vehicle for 40 minutes at 37° C. Control coverslips, both normal and infected monkey kidney cells, were incubated in the vehicle alone. In a few experiments digestion tests were performed on unfixed coverslip preparations.

DNA. Carnoy fixed coverslips were incubated for 10 minutes in 0.01% 1 × crystallized deoxyribonuclease[2] in 0.025 M Veronal buffer containing 0.003 M $MgSO_4$. Control coverslips, both normal and infected, were incubated in the vehicle alone.

Fluorescence Microscopy. The Reichert fluorescence equipment FLUOREX arranged for dark field illumination was used. For observation of acridine orange vitally stained monolayers or preparations fixed and then stained with acridine orange the high pressure mercury lamp was screened with filters BG 12 and 5113 to emit blue-violet light (~ 4500 Å). With eye piece barrier filter OG 1 the transmitted color of nonspecifically stained material appeared green, cellular DNA a brilliant greenish yellow, and cellular and viral RNA flame red against a black background. For observation of slides stained with fluorescent antibody solutions an excited filter 5970 and barrier filter GG 9 were employed.

[1] Pentex Incorporated.
[2] Nutritional Biochemicals Corporation.

Photography. For color photography Kodak high speed ektachrome 35 mm film was used, for black and white Ansco super hypan film.

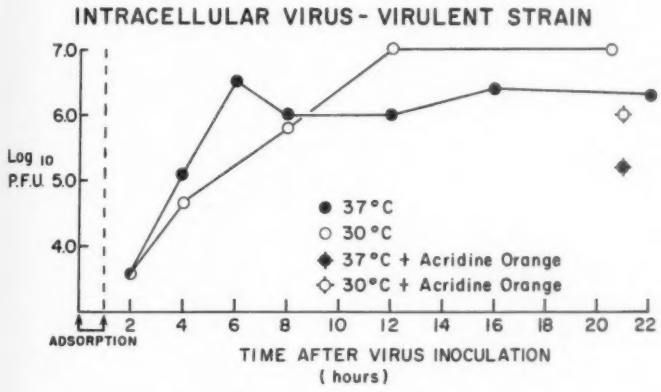
Results

In general there were no significant differences in staining properties either with the acridine orange of fluorescent antibody techniques between the virulent and attenuated strains of poliovirus. The descriptions given below, except where special reference is made, apply equally well to any of the strains of types 1, 2, and 3 tested. In Carnoy-fixed preparations, material identified as DNA by the acridine orange technique, in every case stained a brilliant greenish yellow and was susceptible to DNAase; material identified as RNA stained a brilliant flame red and was susceptible to RNAase.

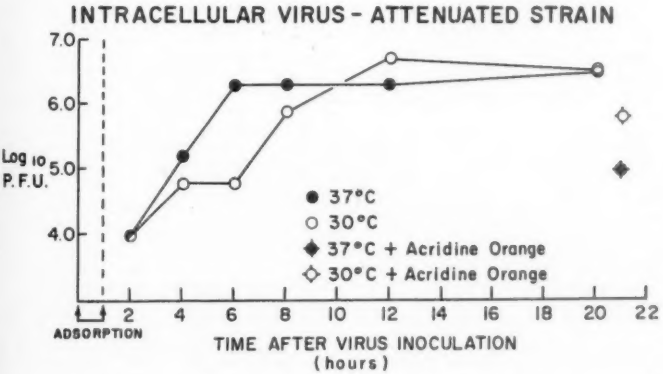
Virus Growth at 37° C. In cysteine medium cytopathic changes were always well established within 6 hours of inoculation. Using the acridine orange technique changes could be detected in the distribution of nuclear DNA as early as 3 hours after inoculation. The appearance of normal monkey kidney cells in cysteine medium by the acridine orange technique is shown in Fig. 1. The nucleus shows an even light green appearance lightly flecked with DNA, the nucleoli exhibit a reddish tinge demonstrating the presence of RNA. The cytoplasm is uniformly flame red. Fig. 2 shows the nucleoli of monkey kidney cells 3 hours after infection. There is a marked clearing evident in the nuclear background and clumping of nuclear DNA which appears to stain very brightly. The nucleoli are stained a brighter red than in the control cultures indicating increased nucleolar RNA activity. There is no detectable increase in the intensity of staining of cytoplasmic RNA. However by 4 hours after inoculation a definite increase in cytoplasmic RNA above the level usually encountered in normal monkey kidney cells (except those undergoing mitosis which stain brilliantly) could be detected in infected cells. Many cells were beginning to elongate at this stage and nuclear clearing with DNA clumping were very marked.

Antigen staining with the fluorescent antibody label was uniformly negative 2 hours after inoculation. By 3 hours particularly in the case of the attenuated strains which were inoculated at a slightly higher multiplicity than the virulent strains a faint but diffuse specific cytoplasmic fluorescence could be detected. In

some cells this fluorescence was located in a bright ring of cytoplasmic antigen surrounding the nucleus (Fig. 10). By 4 hours in all the infected cultures diffuse cytoplasmic fluorescence was well established and brightly fluorescing granules could be detected (Fig. 11).



TEXT FIG. 1. Growth of poliovirus type 1, LSc attenuated strain at 37° C. and 30° C. Titers reached in presence of acridine orange after 21 hours at 37° C. and 30° C. are included.



TEXT FIG. 2. Growth of poliovirus type 2 MEF_1 virulent strain at 37° C. and 30° C. Titers reached in presence of acridine orange after 21 hours at 37° C. and 30° C. are included.

Six hours after inoculation when intracellular virus had reached a maximum (Text Figs. 1, 2) a maximum brilliance in cytoplasmic RNA staining was encountered with acridine orange, usually involving 50% of the cells (Fig. 3). Many cells were beginning to round up, the nuclei becoming eccentric and pyknotic with brilliant and concentrated DNA staining. By 8 hours about 50% of the cells were rounded and marked release of brilliant RNA material was observed in blebs at the cell wall (Fig. 4). This was interpreted as indicating release of poliovirus which is known to reach its maximum at 8 hours in monkey kidney monolayers (16). By this time many of the rounded cells were completely void of RNA and were conspicuous for their shrunken appearance and pyknotic nuclei showing advanced fragmentation of nuclear DNA (karyorrhexis Fig. 7). In the cytoplasm of cells at this stage bright RNA-containing chunks were often found (Fig. 7). It is considered that these represent mainly a breakdown of cellular rather than viral RNA as most of the virus has been released at this stage. However, similar chunks were sometimes encountered in fluorescent antibody

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FIG. 1. Normal monkey kidney cells stained with the acridine orange technique. Note reddish tinge in the nucleoli indicating presence of RNA, flame red cytoplasmic RNA and yellow DNA staining in the nucleus. X 2000.

FIG. 2. Monkey kidney cells 3 hours after inoculation with Y-SK type 2 attenuated virus. Nuclear clearing and clumping of DNA are evident. Nucleoli are stained brightly red. X 2000.

FIG. 3. Monkey kidney cells 6 hours after inoculation with Y-SK type 2 attenuated virus. Brilliant cytoplasmic RNA staining is present in 50% of the cells. Nuclei stain brightly for DNA and are pyknotic. X 900.

FIG. 4. Monkey kidney cells 8 hours after inoculation with LSc type 1 attenuated virus. Note rounded cells full of RNA and eccentric brilliantly stained nuclei. Release of RNA material is evident at the cell walls. X 2000.

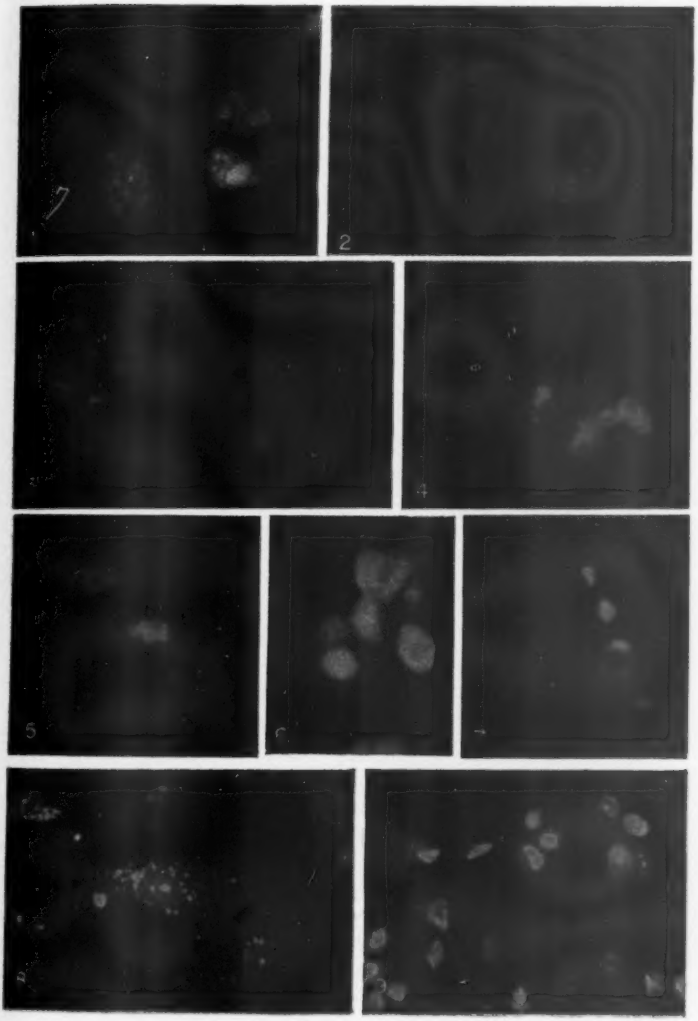
FIG. 5. Monkey kidney cells 8 hours after inoculation with Mahoney type 1 virulent poliovirus. Note 3 dimensional appearance of cells and peripheral RNA staining; also absence of viral release. X 900.

FIG. 6. Infected monkey kidney cells vitally stained with acridine orange fluorochrome. Note rounding of cells and the presence of particulate-like condensations of the dye. X 900.

FIG. 7. Shrunken monkey kidney cells 8 hours after inoculation with Mahoney type 1 virulent poliovirus. Most of the virus has been released from these cells. Note distribution of nuclear DNA material and bright RNA chunks in the cytoplasm. X900.

FIG. 8. Normal monkey kidney cells vitally stained with acridine orange 1/200,000. Note the many inclusions of the dye in the cytoplasm of these cells. X 900.

FIG. 9. Infected monkey kidney cells vitally stained with acridine orange and briefly fixed in alcoholic fixative. Note the absence of acridine orange particles and the bizarre nuclear shapes. X 900.



specimens at this stage so it must be assumed that some antigen remains associated at least for a time with these shrunken cells.

6–8 hours after inoculation also marked a peak in brilliance of fluorescent antibody staining. At 6 hours bright cytoplasmic fluorescence both diffuse and granular could be detected in elongated cells and many cells were already rounded and full of antigen. By 8 hours as many as 50% of the cells in the sheet were rounded and release of antigen, again in blebs, could be detected (Fig. 12). Of particular interest in these specimens was the impossibility of detecting a clear nuclear shadow. This is not taken as evidence of antigen in the nucleus but rather in agreement with Lebrun (18) as due to nuclear degeneration.

Twelve hours after inoculation with both techniques changes indicating a second cycle of multiplication were detected, fresh cells becoming involved in early changes as described above for 3–4 hours after inoculation. From 16–20 hours brilliant rounded cells releasing antigen and RNA material were again at a maximum. The number of exhausted cells containing RNA chunks and degenerating nuclei was particularly high by this stage.

Titrations of intracellular virus at 37° C. (Text Figs. 1 and 2) revealed a gradual increase in the amount of virus reaching a maximum around 6–8 hours when 50% of cells were showing cytopathic changes. This finding was in good agreement with the increases in RNA and antigen staining described above. The level of intracellular virus was maintained as fresh cells became in-

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FIG. 10. Monkey kidney cells 3 hours after infection with LSc type 1 attenuated poliovirus stained by the direct fluorescent antibody technique for viral antigen. Note bright fluorescent staining in a ring around the nucleus in 2 cells at the center of the field. X 1000.

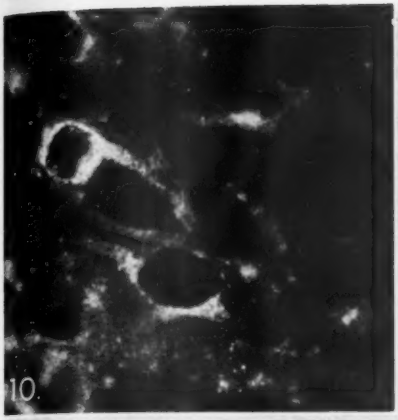
FIG. 11. Monkey kidney cells 4 hours after infection with LSc type 1 attenuated poliovirus. Diffuse and granular fluorescence is found throughout the cytoplasm of the 3 infected cells present. X 1000.

FIG. 12. Monkey kidney cells 8 hours after infection with MEF_1 type 2 virulent poliovirus. Cells are rounded and full of antigen and nuclei are very unclear. Release of antigen in blebs can be observed at the cell walls. X 1000.

FIG. 13. Monkey kidney cells 6 hours after infection with LSc type 1 attenuated poliovirus, incubated at 30° C. Note rounding of cells and faint but specific peripheral antigen. X 1000.

FIG. 14. Monkey kidney cells 8 hours after infection with LSc type 1 attenuated poliovirus, incubated at 30° C. Note swelling of cells, integrity of nucleus and diffuse antigen. X 1000.

FIG. 15. Monkey kidney cells 21 hours after infection with MEF_1 type 2 virulent poliovirus, incubated 20 hours at 30° C. and then placed for 1 hour at 37° C. Note intense fluorescence completely filling the cell and extensive release of antigen. X 1200.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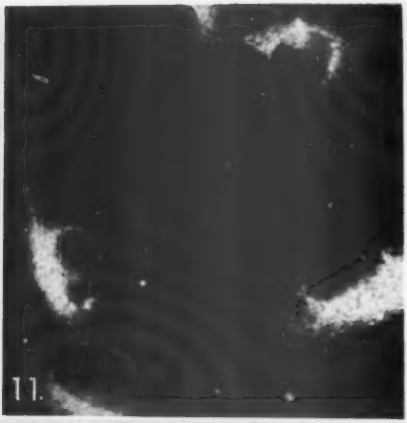
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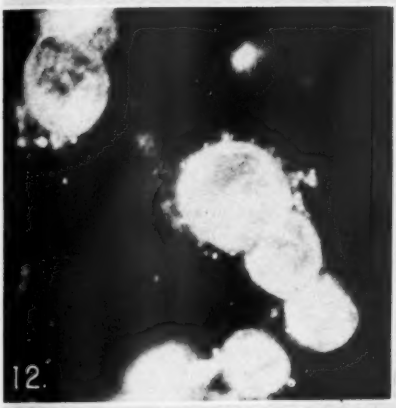
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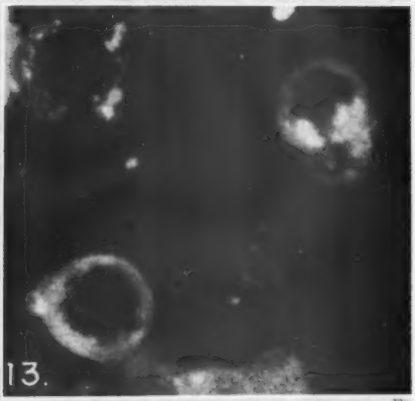
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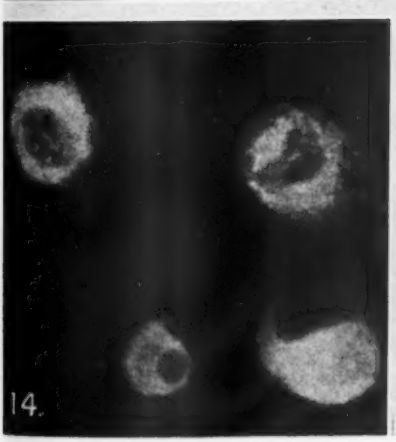
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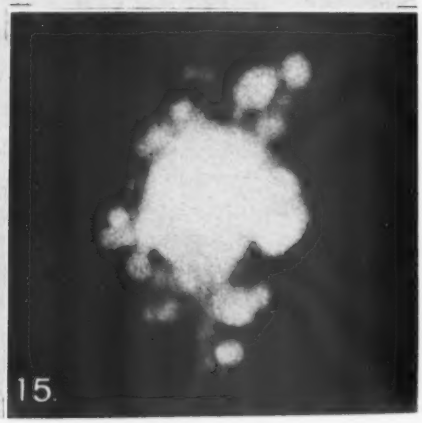
15.

volved until the end of the experiment at about 20 hours when 80 to 95% of the cells showed cytopathic changes. Continued brilliance was observed with both staining techniques during this time.

Virus Growth at 30° C. Morphological changes occurred more slowly in the specimens incubated at 30° C. but the general characteristics of the changes observed were the same. An 8-hour specimen at 30° C. was similar to a 4-hour one at 37° C. and maxima in antigen staining and brilliance of intracellular RNA occurred at 12 hours after inoculation rather than at 6–8. The rounded cells in the 30° C. specimens were always larger than in the 37° C. and were noteworthy for the 3-dimensional effect they presented in the fluorescence microscope. Roizman (30) has shown that growth at 30° C. interferes with release of virus and that maturation is unaffected, and the increased size and shape of the rounded cells encountered here would indicate that these cells are very full of viral material. Fig. 5 shows one such cell using the acridine orange technique. Fig. 13 represents an early stage in 30° C. growth using the labelled antibody technique. The cells round up at about 8 hours (approximately equivalent to a 4 hour specimen at 37° C. when, however, most of the cells are not rounded) and the antigen although faint is located in a definite cytoplasmic ring. Fig. 14 shows a slightly later stage when the cells are swelling and filling with antigenic material.

The persistence of a discrete nuclear shadow (Fig. 14) is in contrast to the 37° C. specimens (Fig. 12). Also during the time the experiment was carried out (20 hours) very few degenerating cells containing chunks of RNA and nuclear material were found. Very little release of virus was observed in specimens incubated at 30° C., however, if such a specimen were placed after incubation at 30° C. in the 37° C. incubator for 1 hour the integrity of the nuclear shadow disappeared and a very striking and brilliant picture of viral release was obtained (Fig. 15). Text Figs. 1 and 2 show the slower growth rate at 30° C. in agreement with the cytochemical and fluorescent antibody findings and indicate the high and persistent levels of intracellular virus maintained in these experiments.

Virus Growth in Presence of Acridine Orange. These experiments were carried out originally to see whether it were possible to visualize poliovirus developing in the presence of acridine

orange, thus tissue culture monolayers were examined when it was known by previous experiment that the titer of intracellular virus was high and the control cells in excellent condition. From 20–22 hours after inoculation was found to be a convenient and satisfactory time. Text Figs. 1 and 2 show the titers of intracellular virus reached in the presence of acridine orange. The approximate 1 log inhibition noticed is probably due to photodynamic inactivation by stray light (12). The higher titer reached for both virulent and attenuated strains at 30° C. is not considered significant.

Fig. 8 shows normal monkey kidney cells grown in the dark in presence of acridine orange. The cytoplasm contains many particle-like condensations of the dye. These cells continued to undergo mitosis and remained healthy in the 1/200,000 dilution of the dye used in these experiments. Fig. 6 shows cells from a specimen containing a high concentration of intracellular virus. All the cells are rounded and contain particles of acridine orange dye. As such cells were never encountered in control specimens it can be assumed that cells containing acridine orange dye are capable of supporting the growth of poliovirus. However, the actual virus particles cannot be visualized by this vital-staining-in-vitro technique. Experiments making it possible to visualize purified concentrates of acridine orange-incorporated poliovirus and some theoretical considerations are being reported separately (24).

The behavior of the acridine orange condensates vitally ingested by normal and infected cells is noteworthy. Their orange color is completely obliterated by alcoholic fixation (Fig. 9) but returns after subsequent staining with fresh acridine orange dye by the standard technique and stands out against the background of cytoplasmic RNA. They are not removed by treatment with ribonuclease while fixed cellular and viral RNA are completely removed. They are completely removed from unfixed cells by 1 hour incubation at 37° C. in McIlvaine's buffer but appear untouched if the buffer contains acridine orange in 1/200,000 concentration. The acridine orange condensates would therefore appear not to be strongly bound to cytoplasmic nucleic acid in vitally stained cells. Their behavior in McIlvaine's buffer with and without acridine orange would indicate an osmotic effect

across the cell membrane based on concentration of the dye on either side.

Discussion

It would appear that both the fluorescent antibody and acridine orange techniques are very sensitive means for following the growth of poliovirus in tissue culture. The limit of resolution of the fluorescent antibody technique is in the vicinity of 5×10^{-15} gm antigen (10) which would correspond to some hundreds of poliovirus particles. Mayor and Diwan (24) have estimated the sensitivity of the acridine orange technique for small RNA viruses to be of the order of 50 particles. However the specificity of the acridine orange method particularly for the study of RNA viruses against a background of cellular RNA is dependent on changes in density and distribution of the nucleic acid and is not as highly specific as the fluorescent antibody technique.

Anderson *et al.* (2) have pointed out that in the case of DNA viruses a relative insusceptibility to DNAase digestion is a constant feature of animal virus infections and that resistance to the nuclease could be abolished by pre-treatment with a proteolytic enzyme. Here we have a useful tool for distinguishing between the host cell and the viral DNA. However, so far no cytochemical method has been found for distinguishing between viral and cellular RNA. Influenza (2), rous sarcoma (13) and poliovirus (34, and present communication) are RNA viruses which in the fixed state have proved susceptible to RNAase. Perhaps a method of fixation which will allow digestion of cellular RNA while leaving viral RNA intact will be developed. Some experiments along this line have been carried out by the present author but without success. However, a combination of the acridine orange technique with that of fluorescent antibody staining is a valuable tool for studying maturation of viruses.

The cytochemical changes reported here agree well with the morphological findings of Reissig *et al.* (28) in their study of poliovirus growth in tissue culture, although the acidophilic intranuclear inclusions observed by them early in infection could not be detected in the present study as these inclusions obviously did not contain viral antigen. The earlier nuclear changes reported here (clearing and clumping of DNA) were never observed during so-called senile pycnosis of cells during starvation.

Lebrun (18) reported detection of antigen by the fluorescent antibody technique 1 hour after inoculation with Brunhilde strain poliovirus at a multiplicity of 10 PFU/cell. The earliest specific changes with the strains tested here at a multiplicity of approximately 5 PFU/cell were 3 hours after infection. The sequence of changes observed agrees well with those reported by Buckley (8, 9) using the indirect fluorescent antibody technique.

Ackermann *et al.* (1) have studied the incorporation of P-32 into RNA during a single cycle of growth of poliovirus in HeLa cells. They found that cytoplasmic RNA increased at a constant rate until the fourth hour when the rate became markedly enhanced and infectious virus appeared in the cytoplasmic fraction. From the amounts of RNA and protein formed, their distribution and the composition of RNA nucleotides they concluded that the major proportion of new material formed was not virally destined. Our cytochemical observations are in good agreement with Ackermann's results, for we found that intracellular RNA staining increased rapidly after the 4th hour reaching a maximum at 6–8 hours and that considerable RNA material remained in cells after liberation of virus. Levintow and Darnell (19) studied the synthesis of poliovirus nucleic acid and protein using a radioactive precursor, adenosine-8-C-14, and found that synthesis of viral protein began at about the same time as maturation but was complete about 1.5 hours before and that the bulk of virus RNA formed no earlier than the protein. Again our results are in complete agreement for we found virus protein (antigen) and RNA were first detected at about the same time (3 hours) and then proceeded to a maximum together.

The cytochemical and fluorescent antibody findings are that viral growth is slower at 30° C. and that virus release but not maturation is inhibited. These results are in good agreement with the biological titrations for virus reported here and also with the experiments of Roizman (30). Thread and rod-like structures similar to those observed under the phase contrast microscope by Tenenbaum (35) during her studies of poliovirus growth at low temperature were never observed.

Summary

The sequence of changes in monkey kidney cells infected with poliovirus has been followed both by cytochemical means (acri-

dine orange staining) and by the fluorescent antibody technique. Changes have been followed closely during a single cycle of infection and beyond, both at 37° C. and at 30° C. At 37° C. both virus protein (antigen) and increased cytoplasmic RNA were detectable 3 hours after infection and both reached a maximum in intensity of staining at about 6–8 hours after inoculation. Parallel titrations of intracellular virus revealed a gradual increase in the amount of virus reaching a maximum again at 6–8 hours and were thus in excellent agreement with the cytochemical and fluorescent antibody findings. Evidence of virus release was observed with both techniques.

Similar results were obtained at 30° C. but intracellular changes occurred approximately 4 hours later than those observed at 37° C. The peak of staining intensity occurred about 12 hours after inoculation. In contrast to growth at 37° C. very little virus release was observed at 30° C. unless the tissue cultures were placed at 37° C. for an additional hour at the end of the incubation period. A brilliant picture of virus release was then observed.

The similarity of behavior in the acridine orange and the fluorescent antibody techniques of both the virulent and attenuated forms of poliovirus indicate that it is not possible to distinguish between them either on cytochemical grounds or by their reaction to labelled antibody.

Acknowledgments

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HAEMOBARTONELLA IN THE MONGOLIAN GERBIL[a]

HAIG H. NAJARIAN[b]

In examining the blood of adult Mongolian gerbils, *Meriones unguiculatus*, for microfilariae of the filarial worm *Litomosoides carinii*, basophilic bodies were noted in smears stained with Giemsa stain. These bodies were found on and apparently in the erythrocytes, and in the plasma. As will be detailed below, these bodies were also present at birth of the animals.

The observations and experiments reported in this paper indicate that these bodies are organisms similar to those associated with bartonelliasis of man and animals. Of the four genera, *Bartonella*, *Haemobartonella*, *Eperythrozoon*, and *Grahamella*, the organisms of this report appear to be most closely identified with *Haemobartonella*. Reaction to splenectomy, response to drug treatment, and cultivation are factors which have been considered. Because of the lack of consensus in this field, the organisms found in the gerbil are tentatively designated as *Haemobartonella sp.*

Materials and Methods

Some of the gerbils were purchased from a commercial breeder and the others were born in the laboratory from the same stock.

Thin smears were made from tail blood, were air-dried, fixed with acetone-free methyl alcohol for 2 minutes, stained 20 minutes with a 10% solution of Giemsa stain in distilled water, and rinsed with distilled water. Alkaline Loeffler's methylene blue (aqueous) was used both as a supravital stain in the examination of fresh blood, and with smears fixed with methyl alcohol.

Blood for culture was obtained aseptically by heart puncture of lightly anesthetized animals, and 0.1 cc samples were inoculated both into NNP blood infusion agar slants (Packchanian, 1959) and Noguchi's semi-solid media. Incubation was at both 31° and 37° C.

[a] From the Helminth Laboratory of the Department of Preventive Medicine and Public Health, The University of Texas Medical Branch, Galveston, Texas. This study was supported by a training grant (2E-66) from the Institute of Allergy and Infectious Diseases, USPHS, National Institutes of Health, under the direction of Dr. J. Allen Scott. Accepted for publication Sept. 21, 1960. Appreciation is extended to Drs. A. Packchanian and L. Anigstein of The University of Texas Medical Branch at Galveston, and to Dr. D. Weinman of Yale University, for their interest in this study.

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Splenectomy was performed aseptically on animals under ether anesthesia. The spleen was excised without ligation of blood vessels and the abdominal incision was bound by wound clips.

Results

Blood smear examinations. Of 38 nonsplenectomized adult gerbils, 37 or 94% were positive for basophilic organisms associated with the erythrocytes of peripheral blood. Giemsa-stained blood smears showed the structures to be pleomorphic but mostly coccoid (Fig. 1). They were located in, on, and among the red cells and stained more heavily when located within the cells. The number of organisms per erythrocyte varied from a few to over fifty. The organisms also stained deeply with Leoffler's methylene blue both supravitally and with fixed blood smears.

Splenectomy. Removal of the spleen of two gerbils was followed 5 to 7 days later by a large increase in the number of organisms and proportion of involved erythrocytes (Fig. 2). At the time of greatest numbers of organisms and for several days later, both animals had an unusual number of enlarged polychromatic erythrocytes, a watery consistency of the blood which clotted very quickly, and evidence of erythrocyte fragmentation, thus indicating an anemic condition. The density of organisms diminished two days after peak numbers and both animals survived the post-splenectomy period and at five months showed pre-splenectomy densities of organisms in the peripheral blood.

Treatment. Both tetracycline hydrochloride and neoarsphenamine were administered intraperitoneally at toxic levels which were lethal to the animals A single dose of tetracycline at 400 mg/kg cleared the blood of organisms in one gerbil at 24 hours and reduced the number of organisms in another animal by about 80% at 5 hours. Single intraperitoneal injections of neoarsphenamine at 400, 100 and 20 mg/kg cleared the blood of organisms in 2, 15, and 24 hours respectively. In all treatments, control animals showed no reduction in parasite density. Whether the activity of these two compounds was only transient cannot be stated since all of the animals died shortly after 24 hours. Nevertheless, the organisms did respond to treatment and neoarsphenamine appeared more active.

Cultivation. Heart blood removed from two gerbils and inoculated into both NNP blood infusion agar slants and Noguchi's

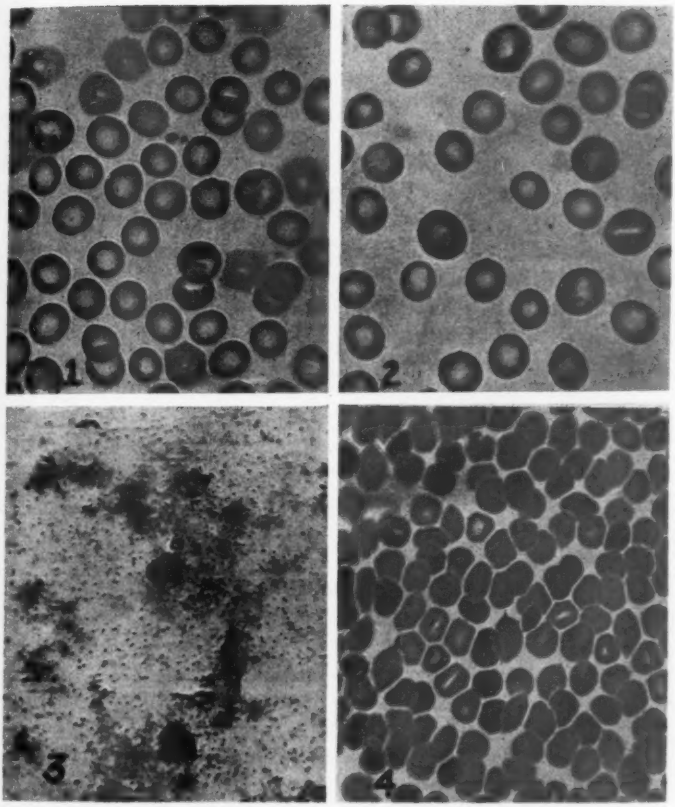

FIG. 1. *Haemobartonella sp.*, Mongolian gerbil blood, non-splenectomized animal, Giemsa stain, X 1380.

FIG. 2. *Haemobartonella sp.*, Mongolian gerbil blood, 7 days after splenectomy, Giemsa stain, X 1560.

FIG. 3. *Haemobartonella sp.*, 48-hour culture, Noguchi's semi-solid medium, Giemsa stain, X 1380.

FIG. 4. *Haemobartonella sp.*, blood of newly-born Mongolian gerbil, Giemsa stain, X 780.

semi-solid media, and incubated both at 31° and 37° C., resulted in growth of the organisms at both temperatures as early as 24 hours. The organisms were coccoid, nonmotile, and occurred singly, as diplococcoid, in beaded formation, and in small and large patches (Fig. 3). Furthermore, many of the organisms were on or in erythrocytes. Growth of the organisms at first was as small, grayish, near-transparent colonies which coalesced into a thin, sticky surface layer. Subcultures from both media were made at 4 and 7 days and the organisms were viable for at least 28 days.

Congenital transmission. Thin smears of peripheral blood were made from a total of 11 gerbils born in the laboratory from three infected nonsplenectomized females. The offspring were less than 24 hours old at the time of examination. Giemsa stains of these blood smears showed all of the offspring to have the same erythrocytic coccoid infection (Fig. 4) as was present in the mother animals. Although no attempt has been made to culture these organisms from newborns, it is believed that these animals could have become infected only at conception or transplacentally.

Discussion

Although Biffi (1903), Gastiaburu (1903), and Barton (1909) reported and described what has come to be known as *Bartonella bacilliformis*, the causative agent of the two forms of Carrion's disease in man, it was not until the studies of Strong *et al.* (1915) that the genus *Bartonella* was created and the etiology of Oroya fever demonstrated. In addition to confirming the earlier observations of the organisms in the erythrocytes of peripheral blood, Strong *et al.* (1915) also found them in fixed tissue cells. The organisms were cultivated by Noguchi and Battistini (1926) and the etiology of Verruga was demonstrated experimentally in monkeys (Noguchi, 1927). The results of the 1937 Harvard Expedition to Peru and a review of other investigations on *B. bacilliformis* was given by Weinman (1944). Kikuth (1931, 1934), and Pittaluga (1938) found that *B. bacilliformis* was not susceptible to treatment with arsenicals. Sandflies of the genus *Phlebotomus* appear to act as vectors in the transmission of the disease (Shannon, 1929; Hertig, 1937). Peters and Wigand (1952) have demonstrated by electron microscopy that *B. bacilli-*

formis has definite bacillus structure, cell wall and membrane, cytoplasm, and unipolar flagella, thus leaving no question of the bacterial nature of this species.

In contrast to *B. bacilliformis*, there has not been complete agreement among various investigators with respect to the nature, classification, and relationships of the other three genera of bartonella-like organisms. The genus *Haemobartonella* was created by Tyzzer and Weinman (1939). Until that time organisms of this genus were considered congeneric with *Bartonella*. The original observations of *Haemobartonella muris* on rodent erythrocytes were made by Mayer and Zeiss (1920), and Mayer (1921). Because of its morphological resemblance to *B. bacilliformis* and its association with anemia in the rats the organism was called *Bartonella muris*. The work of Lauda (1925), and Mayer *et al.* (1926) demonstrated that the "infectious anemia" increased following splenectomy and that it could be reproduced in inoculated animals. Mayer *et al.* (1927) found that the infection was susceptible to treatment with arsenicals. Mayer (1928), and Cannon and McClelland (1928) reported the rat louse *Polyplax* as transmitting agent of *H. muris*. Different results and opinions have existed on the cultivation of *H. muris*, reviews of which are given by Weinman (1935, 1944), and Tyzzer (1942). Degrees of successful cultivation have been reported by Mayer *et al.* (1926), Marmorston-Gottesman and Perla (1932), Battistini and Weiss (1926), and Lawkowicz (1939). Negative results have been published by Pittaluga (1938), Noguchi (1928), Haam *et al.* (1927), Ford and Eliot (1928), and McCluskie and Niven (1934). Recent remarks are still at variance. Wilson and Miles (1955) stated that *H. muris* is cultivable, whereas Peters and Wigand (1955) reported that haemobartonellae have not been successfully cultured. There have been many species of *Haemobartonella* described from rodents and other mammals in many parts of the world, and also structures thought to be haemobartonellae in amphibians, reptiles, and fish (see Weinman, 1944). Although there are several common characteristics between *Bartonella* and *Haemobartonella*, their generic separation on the basis of inability of *Haemobartonella* to multiply in fixed tissue cells and to cause cutaneous eruptions has had support by the work of Peters and Wigand (1955). They have demonstrated that the *Haemobartonella* organisms do not

have morphologic features exhibited by *Bartonella*, such as cell membrane flagella, but rather exist as distinct homogenous particles. They further suggest that haemobartonellae are more distantly related to *Bartonella* than is generally believed.

Members of the genus *Eperythrozoon* are also organisms associated with erythrocytes of rodents and host anemia, especially after splenectomy. Schilling (1928) created the genus, and the early experimental studies on *E. coccoides* were done by him, Dinger (1928), Eliot and Ford (1930), and Perard (1929). This species is also pleomorphic but occurs mostly as coccoid bodies and annular rings, and is found more in the plasma than on the erythrocytes. It has a predilection for polychromatic red cells. Anemia follows splenectomy (Thurston, 1954), and the organisms respond to arsenical treatment (Thurston, 1953). Species of this genus have been reported from various rodents, sheep, and cattle. Lice are believed to play a part in transmission (Eliot, 1936). Cultivation has not been successful, except in embryonated hen eggs (Seamer, 1959). There is not complete agreement as to whether *Haemobartonella* and *Eperythrozoon* have sufficient differences to justify generic or even specific separation. The reports of Schwetz (1934), McCluskie and Niven (1934), and Marmorston (1935), in which mice infected with *E. coccoides* were susceptible to *H. muris* inoculation indicates the distinctness of the two organisms. However, unless the morphological forms of the organisms are followed at close intervals of time after splenectomy, the separation of the identity of the two organisms would seem difficult. Peters and Wigand (1955) are of the opinion that the only real difference between *Eperythrozoon* and *Haemobartonella* is filterability. They further propose that *Eperythrozoon* passes filters because of its predilection for blood plasma, and suggest the two genera as phases of the same organism.

Organisms of the genus *Grahamella* have resemblance to *B bacilliformis* in morphology and location in the peripheral blood, being more uniformly rod-shaped and occurring inside erythrocytes. Graham-Smith (1905) first observed the organisms in erythrocytes of moles. Brumpt (1911) created the genus and described *G. talpae* as type species. Splenectomy is reported not to cause an increase in the number of organisms (Adler, 1930) except in the case of the white rat (Vassiliadis, 1935). Treatment

with arsenicals is not effective (Kikuth, 1934), and the organisms are readily cultivable (Jettmar, 1932; Tyzzer, 1942). In view of the fact that *Grahamella* can be cultivated, the opinion of Wenyon (1926), that the basophilic bodies are probably not organisms seems unjustified. The characteristics of *Grahamella*, such as morphology, location with respect to erythrocytes, reaction to splenectomy and arsenical treatment, and ease of cultivation, seem to permit criteria of difference in relation to *Haemobartonella* and *Eperythrozoon*. However, it is not always an easy matter to ascertain the identity of the organism. Tyzzer and Weinman (1939) reported on *Haemobartonella*, only to conclude later (Tyzzer, 1942) that they were dealing with a mixed infection on *Haemobartonella* and *Grahamella*. Noguchi (1928), and Adler (1930) indicated that *Haemobartonella* and *Grahamella* could be distinguished on morphological grounds. Due to the high degree of polymorphism of *Haemobartonella*, this view is questioned. The culturing of bartonella-like organisms in embryonated hen eggs (Seamer, 1959) should prove useful in the reinvestigation of the identity of *Haemobartonella* and *Eperythrozoon*, and *Grahamella*. Although the systematic position of these organisms with respect to rickettsia, viruses, and bacteria is not clear, there is little doubt that their features justify their being considered as microorganisms.

It is concluded that the microorganisms of the present study be tentatively designated as *Haemobartonella sp.* On the basis of morphology, relation to erythrocytes, and cultivation they have less relation to *Eperythrozoon*, than to *Haemobartonella* and *Grahamella*. Although they more closely resemble *Haemobartonella* than *Grahamella* in morphology, their location with respect to erythrocytes coincides both with the intracellular nature of *Grahamella* and the cell surface of plasma features of *Haemobartonella*. Reaction to splenectomy and response to arsenical treatment are similar to *Haemobartonella*. Cultivation places the organisms perhaps closer to *Grahamella* than to *Haemobartonella*.

Adler (1930) has reported *Grahamella meriones* from *Meriones tristami*, a common rodent of the Levant. He stated that infected erythrocytes were rare and appeared bluish with Giemsa stain. In contrast, the *Grahamella* of infected cells were numerous, almost entirely filled the erythrocytes, and did not increase

in numbers following splenectomy. Infected erythrocytes of non-splenectomized gerbils (*M. unguiculatus*) of the present study were not rare, and in most cases the involved cells stained normally with Giemsa stain, except after splenectomy. Furthermore, the organisms from the Mongolian gerbil usually did not completely fill the erythrocytes, and markedly increased after splenectomy.

If the conclusion is correct that the organism of the present study is a species of *Haemobartonella*, then as far as has been determined, the Mongolian gerbil constitutes a new host for this genus of microorganisms. Furthermore, this study constitutes another report on the successful cultivation of a species of *Haemobartonella*. Since no information was obtained on pathogenicity and host specificity, it appears best to withhold specific designation of the organism at this time.

Some of the gerbils of this study were combed for lice but none was found. In receiving a shipment of 40 gerbils from a commercial breeder, blood smears from five random animals showed all to be infected with *Haemobartonella sp.* It is a striking fact that the infection rate appears to be so high. If the indication that congenital transmission of the organisms takes place is confirmed, then not only would this represent a situation in which a microorganism in a female mammal is transmitted to its offspring before birth, but there would be added significance in that perhaps the high incidence of this organism in the gerbil would be a factor to be considered in its utilization as an experimental laboratory animal.

It is of interest to note that congenital transmission of *B. bacilliformis* in humans has been reported to occur (Monge, 1912; Malpatida, 1937; Otalora, 1939). However, the studies of Mayer *et al.* (1927), and Severi (1937), have indicated that such transmission to rodent offspring has not been observed. Future work is planned to confirm the apparent congenital transmission of *Haemobartonella sp.* in the Mongolian gerbil, and to elucidate the possible mechanisms involved.

Summary

An examination of the peripheral blood of the Mongolian gerbil, *Meriones unguiculatus*, purchased commercially and laboratory-reared, showed almost 100% infection with a bartonella-

like organism. After splenectomy, the number of organisms increased to a peak at 5 to 7 days, followed by a transient anemia and survival of the animal. The organisms responded to intraperitoneal treatment with tetracycline and neoarspheramine at toxic levels. Organisms from gerbil heart blood grew when inoculated into NNP blood infusion agar slants and Noguchi's semisolid media and incubated at both 31° and 37° C. The organism is tentatively designated as *Haemobartonella sp.* The characteristics and relationships of *Bartonella*, *Haemobartonella*, *Eperythrozoon*, and *Grahamella* are discussed. The peripheral blood of all of 11 newly-born gerbils was infected with *Haemobartonella sp.* and indications are that the high infection rate in the gerbil may be associated with congenital transmission of the organism.

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HISTOCHEMICAL DEMONSTRATION OF SUCCINIC DEHYDROGENASE SYSTEM IN TISSUE CULTURE

KAZUO OGAWA AND MICHIO OKAMOTO[a]

Most of the cytochemical studies of the succinic dehydrogenase system (SDS), or succinate-tetrazolium reductase system, have been performed on tissue slices or sections and there has been very little research done on individual cells cultivated *in vitro* (2, 3), although tissue culture is known to be one of the best methods of observing the metabolism of single cells.

This investigation compares the supravital method (6), the agar overlay method (2) and the cold acetone fixation method (4) of demonstrating SDS activity in terms of maintenance and intracellular localization of enzymatic activity in puppy cerebellum cell cultures. The results of studies of these cells were compared with those obtained by Ogawa and Zimmerman (6) in slices of normal C3H mouse brain.

Materials and Methods

Cultures: The cerebellar cortices of new-born puppies were cultured in vitro by a roller tube method (1, 5). The culture medium consisted of 50% human ascitic fluid, 45% Gey's balanced salt solution and 5% chick embryo extract with the addition of 3 mg glucose and 1000 units of penicillin per ml (9, 10). The culture medium was changed once a week, and 3 to 15 day-old cultures were used for histochemical demonstration of SDS.

Histochemical demonstration of SDS: A. Supravital method: Culture specimens were removed from the roller tubes and unfixed unfrozen specimens were incubated directly in the substrate medium under aerobic conditions without washing. The incubation medium was prepared from equal volumes of 0.1% solution of nitroneotetrazolium chloride, 0.1 M phosphate buffer, pH 7.6, 0.1 M sodium succinate in buffer, and distilled water, without any coenzymes. Specimens were incubated in the incubation medium at 37° C. for 1–2 hours or sometimes overnight. Then they were rinsed briefly in phosphate buffer, fixed in 10% neutral formalin for several hours, washed in water and mounted in a glycerine-jelly.

Control specimens were incubated in the medium without sodium succinate, in the medium plus 0.1 M sodium malonate, and in the medium plus 0.2% potassium cyanide. B. Both agar overlay method (2) and cold acetone fixation method (4) were also used and compared with the supravital method.

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Results

Supravital method: Neurons, perhaps Purkinje cells, revealed the highest SDS activity as evidenced by massive deposits of formazan granules throughout the perikaryon and also in dendrites (Fig. 1). No definitive conclusions were obtained in regard to oligodendroglia, since it was rather difficult to distinguish oligodendroglia in tissue culture, particularly of the cerebellum. However, occasionally satellites showed some activity (Fig. 2). Satellites are defined by position.

Various types of astrocytes (Figs. 3, 4) and fibroblasts (Figs. 5–8) showed moderate SDS activity, and there appeared to be no direct correlation between SDS activity and types of astrocytes as defined by Ogawa (8). Formazan granules were observed even in the processes connecting fibroblasts (Fig. 8). Microglia (Fig. 9) and macrophages showed varying degrees of activity. No SDS activity was observed in the nuclei of any of the cells. In the Golgi zone, formazan granules were much fewer than in other areas of the cytoplasm (Figs. 3–5), and a lower SDS activity was observed in areas containing lipid granules (7, 8) than in those without granules (Fig. 7) It was interesting to find structures resembling mitochondria (Figs. 3, 6) following incubation overnight.

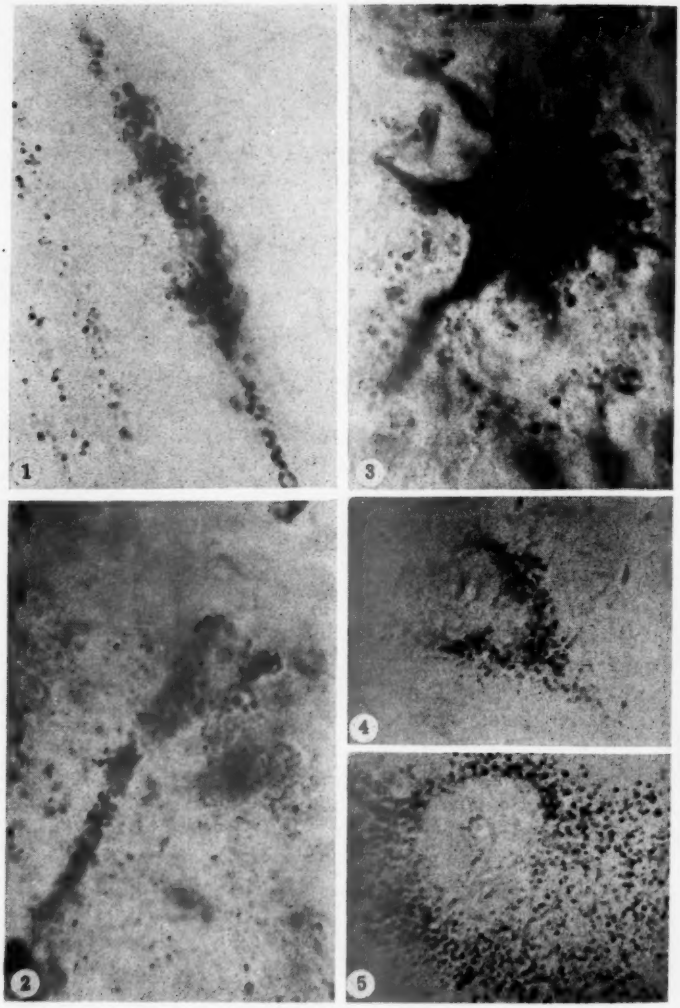
Sodium malonate inhibited SDS activity completely but potassium cyanide lowered enzymatic activity slightly in the concentrations used.

Agar overlay methods: Enzymatic activity was somewhat less apparent by this method than by the supravital method. Formazan granules in the cytoplasm were rather coarse and structures resembling mitochondria were not seen even after prolonged incubation.

Cold acetone fixation method: The SDS activity was completely lost after cold acetone fixation (2° C., 10 minutes).

Discussion

As reported previously by Ogawa and Zimmerman (6), fresh unfixed specimens were most satisfactory in terms of maintenance and precise intracellular localization of enzymatic activity in slices or sections. This was also the case with cultured ma-



FIGS. 1–9. Histochemical demonstration of succinic dehydrogenase system (SDS) in cells cultivated in vitro. Nitroneotetrazolium chloride was used as an electron acceptor. Materials used in this investigation were obtained from cultures of the cerebellar cortices of new-born puppies.

FIG. 1. Purkinje cell from a 15 day-old culture. Formazan granules are revealed as black granules. Note massives deposits of formazan granules in the perikaryon and dendrites. X 1000.

FIG. 2. More or less degenerating neuron with an accompanying satellite in a 15 day-old culture. X 1000.

FIG. 3. A fusiform astrocyte from a 7 day-old culture. Note structures resembling mitochondria in the cytoplasm and processes. X 1000.

FIG. 4. A standard astrocyte from a 6 day-old culture. Note the scarcity of formazan granules in the Golgi zone. X 1000.

FIG. 5. A fibroblast in a 10 day-old culture. The scarcity of formazan granules in the Golgi zone is noted. X 1000.

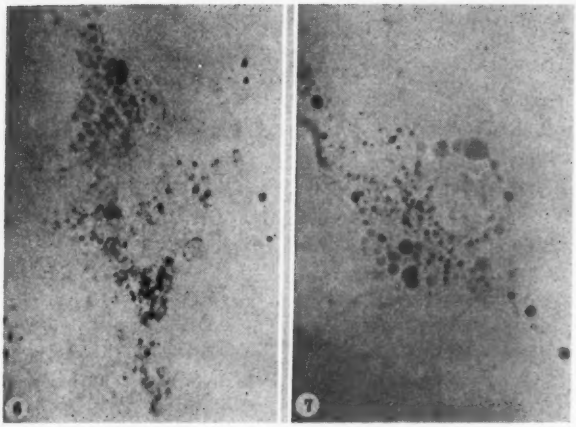


FIG. 6. A fibroblast from a 7 day-old culture. Structures resembling mitochondria are prominent in the cytoplasm. X 1000.

FIG. 7. A fibroblast from a 7 day-old culture. Areas of cytoplasm with lipid granules show a lower SDS activity, indicating a lower rate of oxidative respiration, than areas without granules. X 1000.

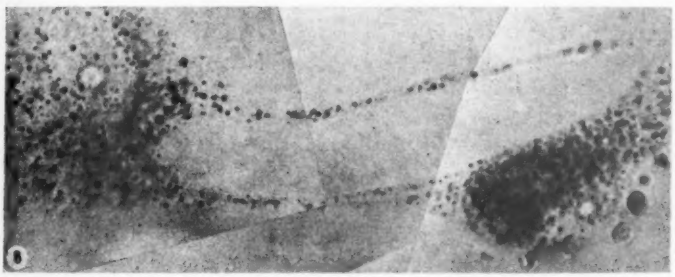


FIG. 8. Two fibroblasts from a 7 day-old culture. Note the presence of formazan granules in the processes connecting two cells. X 1000.

FIG. 9. Microglia in a 10 day-old culture. X 1000.

terials and it was concluded that the supravital method was best for a histochemical demonstration of SDS activity in tissue cultures.

Neurons in the cerebellar cortices revealed the highest SDS activity in tissue culture also (Fig. 1), as in slices (6). In slices oligodendroglia, particularly satellites defined only by position showed higher SDS activity than astrocytes. However, in cultured materials it was somewhat difficult to compare the activity, since morphological differences between oligodendroglia and young astrocytes were obscure, although satellites could sometimes be identified from the characteristic position near neurons in explants (Fig. 2). Various types of astrocytes (8) and fibroblasts revealed moderate SDS activity (Figs. 3–8), and microglia as well as macrophages showed varying degrees of activity.

It was interesting to find a lower SDS activity in areas of cytoplasm containing lipid granules than in areas without granules in both astrocytes and fibroblasts (Fig. 7). This suggests that the cells with many lipid granules possess lower SDS activity than those with few or no granules. The lower SDS activity, indicating a lower rate of oxidative respiration, was compatible with the finding of thinner and fewer mitochondria in the areas with lipid granules (8). By and large, however, the mechanism of lipid granule formation and the metabolic role of granules are yet unknown.

Summary

1. The supravital method, the ager overlay method and the cold acetone fixation method for the demonstration of the succinic dehydrogenase system were applied to cultures of cerebellar cortices, and it was concluded that the supravital method was most satisfactory in terms of maintenance and precise intracellular localization of enzymatic activity.

2. In tissue cultures, neurons revealed the highest activity of succinic dehydrogenase system. Satellites, occasionally found, had some activity. Various types of astrocytes, and fibroblasts revealed moderate activity, while microglia and macrophages showed varying degrees of activity.

3. A lower activity of the succinic dehydrogenase system, indicating a lower rate of oxidative respiration, was observed in areas of cytoplasm containing lipid granules than in those without granules in both astrocytes and fibroblasts cultured *in vitro*.

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A FUNGAL PIGMENT WITH CERTAIN UNIQUE PROPERTIES[a]

G. E. Peterson, R. Livesay, and Helen Futch[b]

A review by Raistrick (1950) described the many classes of pigmented compounds that occur in fungi. However, none of these has extraction characteristics, absorption maxima and pH indicator properties associated with certain anthocyanidins of flowering plants.

One of our soil isolants, UHPRP 1259, has septate mycelium, small, elongated, colorless conidia, and white aerial and red vegetative mycelium. When exposed to NH_4OH fumes the latter turns blue-violet; this reaction is used to detect anthocyanins and anthocyanidins. The organism was grown in shaken flask cultures and media factors affecting pigment production were noted. *N*-amyl alcohol: acetophenone (2:1) extracts of whole broth were tested for optical density at 500 mμ to obtain estimates of pigment production. Complex organic nitrogen sources failed to support any detectable pigment production. A "tomato soup" appearing broth with maximal pigment production was obtained in 96 hours at 30° C using a salts-glucose medium of the following composition: KH_2PO_4, 1.0g; K_2HPO_4, 1.0g; $MgSO_4$: $7H_2O$, 0.4g; NaCl, 0.2g; $Fe_2(SO_4)3$, 0.02g; $MnSO_4$, 0.2g; $(NH_4)_2SO_4$, 0.4g; glucose, 10.0g; and water, 1000 ml. Autoclaved at 121° C for 30 minutes. This process was scaled into a 3-liter pilot scale culture using the New Brunswick Scientific Co. fermentor Model FS-305. Sterile aeration was maintained at 1000 cm^3/min and the agitation was kept constant with the 2-baffle agitator set at 150 rpm. After 96 hours at 30° C pigment yields of 100–150 μg/ml were obtained.

Sintered glass filtration caused the red, whole broth to yield a clear, uncolored liquid phase. The red mycelium turned blue-violet when treated with NH_4OH and back to red with HCl.

Benzene, ethyl ether, petroleum ether, and *n*-butanol were completely ineffective for the extraction of the red-coloring mat-

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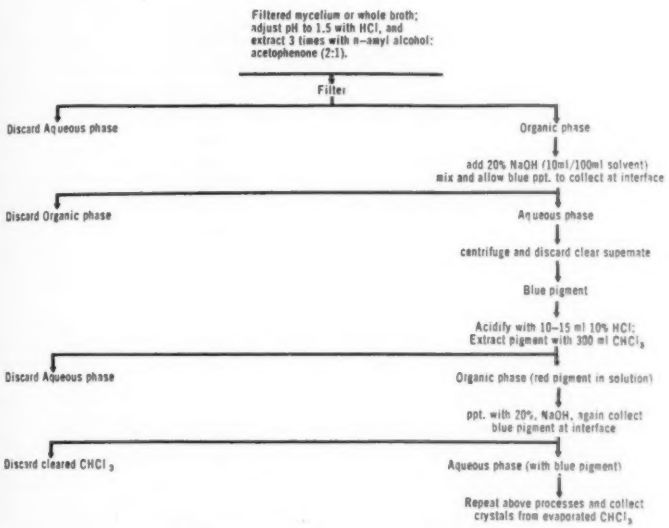
ter. Only when the broth was adjusted to pH 1.5 and extracted with an equal volume of *n*-amyl alcohol: acetophenone (2:1) could nearly quantitative pigment extraction be achieved. Table 1 outlines the procedure used to crystallize the pigment from these extracts. A 3-liter culture yielded 178.5 mg of pigment.

TABLE 1

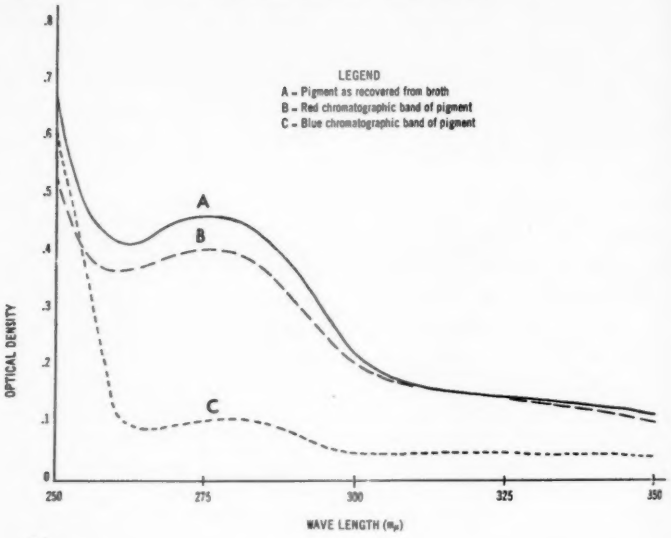

Filtered mycelium or whole broth; adjust pH to 1.5 with HCl, and extract 3 times with n-amyl alcohol: acetophenone (2:1).
Filter
Discard Aqueous phase
Organic phase
add 20% NaOH (10ml/100ml solvent) mix and allow blue ppt. to collect at interface
Discard Organic phase
Aqueous phase
centrifuge and discard clear supernate
Blue pigment
Acidify with 10–15 ml 10% HCl; Extract pigment with 300 ml $CHCl_3$
Discard Aqueous phase
Organic phase (red pigment in solution)
ppt. with 20% NaOH, again collect blue pigment at interface
Discard cleared $CHCl_3$
Aqueous phase (with blue pigment)
Repeat above processes and collect crystals from evaporated $CHCl_3$

Extraction procedure for fungal pigment

The crystals were dissolved in chloroform and chromatographed on a celite: silicic acid (2:1) column. A fast moving red and a tightly absorbed blue band were noted. When the latter was eluted with HCl it turned red; an eluate of the former became blue-violet on treatment with NaOH.

Chloroform solutions of the two eluted bands were compared spectrophotometrically with the crude crystals. Figures 1 and 2 show that the three solutions had similar absorption peaks at 280 and 520 mμ. Schou (1927) noted a quite similar pattern of absorption with some anthocyanidins. None of the available data describing light absorption by fungal pigments shows this pattern.

Table 2 lists some of the physical and chemical properties of


LEGEND
A – Pigment as recovered from broth
B – Red chromatographic band of pigment
C – Blue chromatographic band of pigment
A
B
C
OPTICAL DENSITY
.8
.7
.6
.5
.4
.3
.2
.1
0
250
275
300
325
350
WAVE LENGTH (mμ)

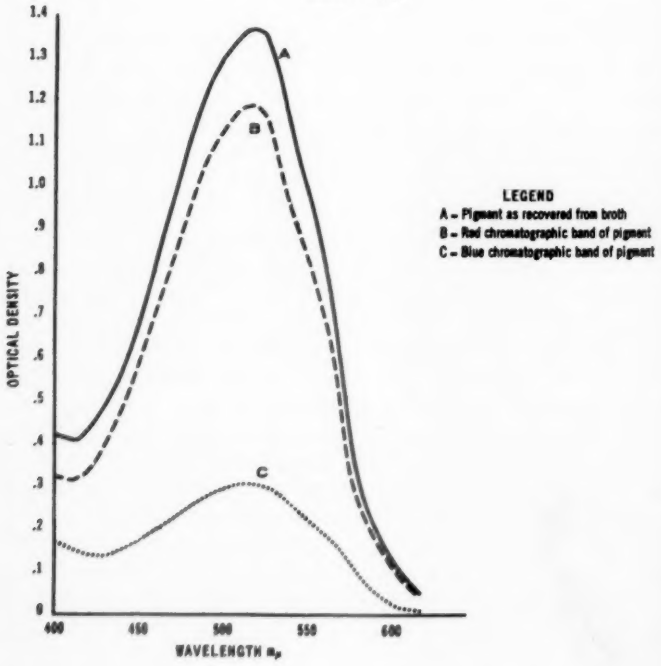

A
B
C
OPTICAL DENSITY
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
400
450
500
550
600
WAVELENGTH mμ
LEGEND
A – Pigment as recovered from broth
B – Red chromatographic band of pigment
C – Blue chromatographic band of pigment

TABLE 2

COLOR: Brownish red. MICROSCOPIC: Long thin needles.
MELTING POINT: not obtainable; Decomposes at approx. 280 – 300° C.
OPTICAL ROTATION: solutions too dark to determine.
MOLECULAR WEIGHT: Insoluble in solvents tested for this determination.
FLOURESENCE: Negative in glacial acetic acid (no quinizarin compound indicated).
INDICATOR PROPERTIES: Red in acid, blue–violet in base.

SOLUBILITIES

SOLUBLE IN:	$CHCl_3$	n–amyl alcohol: acetophenone (2:1)
	Conc. H_2SO_4	Glacial acetic acid
	Boiling 20% NaOH	
INSOLUBLE IN:	Dilute H_2SO_4	$NaHCO_3$
	Ethyl ether	$NaCO_3$
	Ethanol	Cold NaOH
	Benzene	Acetone
	Petroleum ether	Camphor
	Methanol	$Na_2S_2O_4$
	Cyclohexanol	$Na_2S_2O_4$ + NaOH

CHEMICAL ANALYSIS (one determination)

Carbon 61.25	Methoxyl 9.95
Hydrogen 4.61	Nitrogen .4
Oxygen 27.04	Ash 2.19

OTHER CHEMICAL TESTS

Nitrogen – Neg. Benzenoid Structure – at least Bicyclic Anthrone test – Neg.
Hot alkaline hydrolysis – no phenol compounds identified

Physical and chemical properties of fungal pigment

this pigment. The insoluble nature of the substance has complicated these determinations. For want of a suitable solvent no molecular weight has been determined; hence, any consideration of chemical structure would be speculative. The solubilities, C:H:O ratio, and much of the other data are inconsistent with those of known fungal pigments. However, since hot, alkaline hydrolysis failed to reveal any known phenols, the presence of an anthocyanidin is doubtful. Furthermore, negative results were obtained from a series of chemical tests designed by Robinson and Robinson (1931) to identify the known anthocyanidins.

Hence, even though the extractability, absorption spectra and indicator properties of this newly discovered mold pigment resemble those of the anthocyanidins, —its other somewhat unique properties must be more completely investigated before a conjecture of its structure can be made.

FIG. 1. Absorption of ultraviolet light by crude and chromatographed pigment fractions.

FIG. 2. Absorption of visible light by crude and chromatographed pigment fractions.

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YEAST ALCOHOL DEHYDROGENASE—RABBIT ANTIBODY INTERACTION: ENZYMATIC AND TURBIDIMETRIC MEASUREMENT OF AG-AB REACTION[a]

HELMUT M. REDETZKI[b]

Enzymes inducing antibody (Ab) formation can be distinguished from other antigens (Ag's) of protein character when their specific activities are inhibited in the course of Ag-Ab interaction. Such systems offer an additional criterion for immunological investigation which is based on convenient *in vitro* techniques of enzyme assay. However kinetic and quantitative data obtained by enzymatic methods differ considerably according to the type of inhibition involved.

For the interpretation of enzyme-antienzyme interaction it may be considered that two different reactions are superimposed during the measurements: the enzymatic catalysis of conversion of substrate and the immunological formation of the Ag-Ab complex. Changes in the enzymatic rate of reaction which are not caused by the immunological process should be recognized and corrections must be applied for their elimination or extrapolation.

In order to relate results of enzyme-antienzyme studies to basic knowledge and progress in immunochemistry, two important questions remain to be emphasized: 1) which phase in the Ag-Ab interaction is measured with the inhibition reaction, and 2) what conclusions can be drawn from enzyme-antienzyme investigations?

The present report is a part of investigations dealing with some characteristic interactions in the yeast alcohol dehydrogenase (YADH)—anti-YADH system (9, 5). The enzymatic method

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is analyzed and the attempt is made to relate the findings with a different immunological technique, the turbidimetric measurement of Ag-Ab reaction.

Materials and Methods

Immunization and antiserum preparation: Rabbits were immunized by intravenous and intramuscular injections of highly purified YADH (crystallized YADH purchased from Worthington Biochemical Corporation was recrystallized one or two times from ammonium sulfate according to its specific activity). Dosages for the injections ranged from 1.5 mg of enzyme protein with the first injection to 5.0 mg with the booster shot. The rabbits were bled by heart puncture 7–9 days after the last injection. The sera of the rabbits with the highest precipitin and enzyme inhibition titers were partly purified individually by repeated precipitation in ⅓ saturated ammonium sulfate solution, finally suspended in 40% saturated ammonium sulfate, and kept in the refrigerator at 5° C. Prior to use the antibody preparation was dialyzed in the cold for 24 hours against 0.067 *M* phosphate buffer, pH 7.4 or saline.

Enzymatic technique: Enzyme activity was measured with the spectrophotometric technique of Warburg (14). Components of the test system were phosphate buffer 0.067 *M*, pH 7.4 (if not indicated otherwise), ethanol 0.30 *M*, diphosphopyridine nucleotide (DPN) 0.0012 *M* and semicarbazide 0.02 *M*. The relative enzyme activity was expressed as increase in optical density in the stated time interval, the inhibition in per cent was calculated from corresponding activities of tests with Ab and a blank without Ab (a similarly prepared serum fraction of an unimmunized rabbit was used in equal quantities for the blank test). Reaction temperature was 25° C. Readings were made at a wavelength of 340 mμ or 366 mμ (mercury line).

Turbidimetric technique: The test solution, containing the same components as in the enzymatic assay, was filtered through a sintered glass filter in order to reduce light scattering by particulate impurities.

Transmission measurement: Measurement of turbidity by light transmission was performed at 366 mμ in the Eppendorf photometer,[c] light path 4 cm. The optical density ($\log \frac{i_o}{i}$) was used to indicate turbidity values.

Nephelometric measurement: Nephelometric measurements were performed with the nephelometric attachment of the Eppendorf photometer. Incident light 400–600 mμ. The intensity of scattered light was measured at an angle of 90° and compared with a turbidity standard. Readings were taken in turbidity units.

Results

Early phases of Ag-Ab reactions can be readily measured with enzyme-antienzyme systems. It is, however, necessary to evalu-

[c] Netheler and Hinz, Inc., Hamburg, Germany.

ate factors which may interfere in a nonspecific way. This is of special importance when reaction rates are compared.

Reaction rates of formation of ADH—anti-ADH complex

Enzymatic measurements of Ag-Ab reaction rates were performed in two ways: 1) the enzymatic reaction was initiated together with the Ag-Ab reaction, or 2) the enzymatic reaction was started at different time intervals after initiation of the Ag-Ab reaction. The first procedure was called the "zero-time method" and the second the "preincubation method." The tests for both estimations were carried out in a total volume of 2.08 ml; concentrations of the Ab fraction were 290 μg protein/ml, enzyme 0.4 μg/ml (analyzed by biuret reaction). Reduction of DPN in presence of substrate was recorded spectrophotometrically at 366 mμ, light path 1 cm, according to the reaction:

$$\text{ethanol} + \text{DPN}^+ \underset{\rightleftharpoons}{\overset{\text{YADH}}{}} \text{acetaldehyde} + \text{DPNH} + \text{H}^+$$

Zero-time method: The cuvettes contained buffer, DPN, ethanol and Ab or normal globulin fraction respectively. The Ag-Ab reaction and the enzymatic reaction were started simultaneously by addition of YADH (the enzyme was previously diluted 1:500 in ice cold pyrophosphate buffer 0.05 *M*, pH 7.4 and the reaction was initiated exactly 2 minutes later by mixing 0.03 ml of the diluted enzyme with the contents of the cuvette. Micro precision pipettes were used in this step. The accuracy of timing the starting point was estimated to be ± 2 sec). The increase in optical density was measured every 15 seconds during the early reaction period and later every minute. The inhibition curve was calculated from corresponding activity rates in Ab and blank tests (11).

Preincubation method: The cuvettes contained buffer, DPN and Ab. The Ag-Ab reaction was started by addition of diluted ADH as described above. The enzymatic reaction was initiated with substrate added to different cuvettes 1, 2, 3, 5 and 9 minutes after the start of the Ag-Ab reaction. The optical density was measured every 15 seconds over a period of 90 seconds only. The increments in optical density in the first 15 seconds were used for the calculation of the inhibition. The results of these measurements are demonstrated in Fig. 1.

Differences of inhibition rates with preincubation and zero-

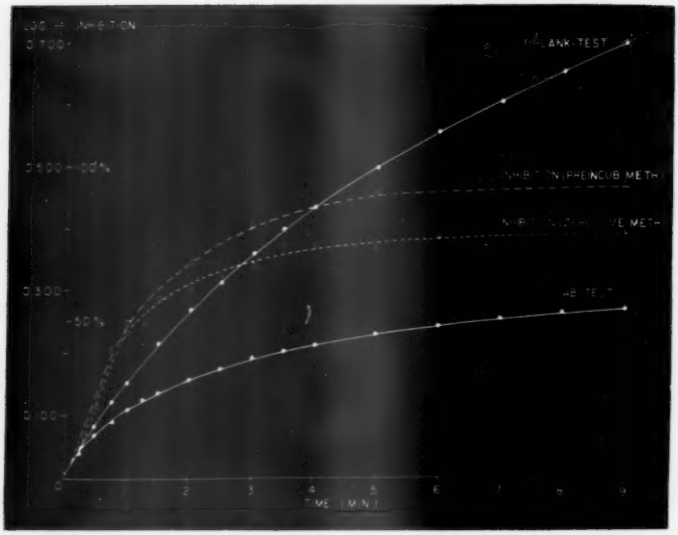

FIG. 1. Enzymatic inhibition of YADH by rabbit serum antibodies: increase in optical density at 366 mμ is plotted against time. The inhibition curves are obtained from measurements with the "preincubation method" and the "zero-time method." Enzyme activity and inhibition are calculated from corresponding reaction rates of tests with Ab and without Ab respectively. (Concentrations, see text.)

time methods. According to arrangement of the measurements significant differences of inhibition rates were observed. Higher values were obtained when Ag and Ab were preincubated in the absence of substrate. For the elucidation of this effect two possibilities had to be considered: a) the formation of Ag-Ab complex involves a competitive mechanism in which substrate and Ab combine with the same site of the enzyme, and b) the accumulation of a reaction product decreases the reaction rate.

Competition between Ab and substrate for the same site of the enzyme molecule could be eliminated. The maximum inhibition measured with the preincubation method was unaltered over a 1:20 concentration range of ethanol. Likewise no evidence was found for competition between Ab and coenzyme when DPN concentration was changed.

The concentration of reaction product was considerably higher in the zero-time method in which the enzyme activity was meas-

ured continuously, than in the preincubation method in which the calculation of inhibition rates was based on initial velocities. Since the presence of semicarbazide in the test system prevents accumulation of acetaldehyde, the decline of the enzymatic reaction rate in the blank test (see Fig. 1) is due mainly to formation of YADH-DPNH complex (3). In order to analyze the implications of this effect on the enzymatic method, experiments were carried out to measure inhibition rates with the reverse reaction using acetaldehyde and DPNH as substrate and coenzyme respectively (the data are not directly comparable with those given in Fig. 1 because the higher rate of reaction necessitated reduction of the enzyme and Ab concentrations). The inhibition at the end of the first, second and third minute was 46%, 67% and 77% in the zero-time method, and 50%, 71% and 75% in the preincubation method. The deviation of these values is within the range of experimental error. It can therefore be concluded that the difference between both methods (in the forward reaction) is "unspecific" and is sufficiently explained by the YADH-DPNH association.

Kinetics of the inhibition reaction

Analyzing the kinetics of the inhibition reaction from the data recorded in Fig. 1, one notices that maximal inhibition was obtained in about 5 minutes. The half maximal inhibition was reached in only 45 seconds. The rate of enzyme inhibition by the serum Ab can be characterized as a second order reaction (or sum of second order reactions): a linear function is gained when the reciprocal of the unreacted enzyme fraction (free enzyme) is plotted against time (Fig. 2). The data obtained with the preincubation method fit closer in this relationship than those gained with the zero-time method.

The influence of increasing Ab concentration on the maximal inhibition was studied under conditions in which Ag concentration was kept constant (1.22 μg enzyme/ml). The preincubation method was applied for the estimation of the apparent end-point of the reaction. Total inhibition of the enzyme was approached asymptotically with effective increase of maximal inhibition in the range of low Ab concentration, leveling off with higher concentrations. The highest inhibition obtainable in Ab excess was 97% (see Fig. 3).

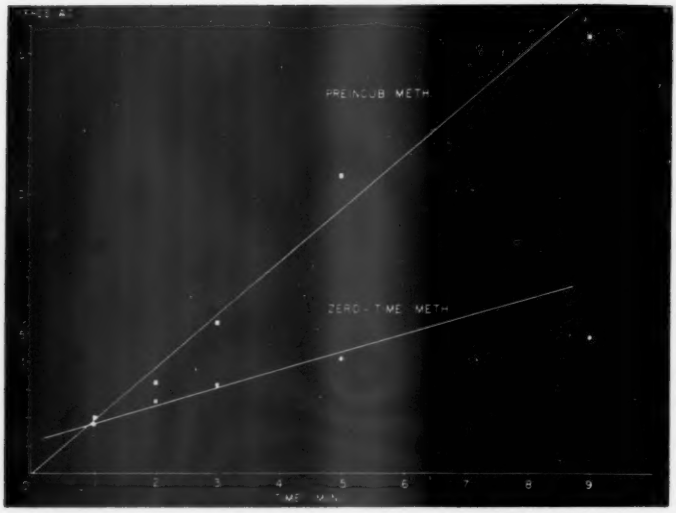

FIG. 2. Inhibition reaction plotted as a second order function: values taken from FIG. 1, concentrations see text. Free Ag is equal to free enzyme (uninhibited fraction of YADH).

The effects of variation of Ag concentration could be measured enzymatically over a limited range only because of technical difficulties in recording fast increases in optical density and unfavorable equilibrium of DPN-DPNH concentrations with high reaction rates. Increase of amount of enzyme at a constant Ab level (30 μg protein, gamma globulin fraction) resulted in a decrease in maximal inhibition (see Fig. 3). The function is comparable with a neutralization phenomenon but the limited range or measurement does not allow its mathematical characterization.

Decline of maximal inhibition in extreme Ab excess was noticed in preliminary studies with antiserum but was absent when the experiments were carried out with the purified gamma globulin fraction. Reversibility of the inhibition reaction (dissociation of the enzyme-Ab complex) was however observed when Ag and Ab were preincubated and the residual enzyme activity was tested after dilution of the sample with buffer.

Comparison of enzymatic and turbidimetric measurements of Ag-Ab reaction. To obtain measurable inhibition effects, the Ab concentration has to be in excess of the enzyme concentration.

This became evident from estimations of the Ab content of antiserum preparations with the quantitative precipitin test (Heidelberger technique). In the range of Ab excess (molar Ag: Ab ratios for these experiments were estimated to be around 1:20 to 1:100), rapid aggregation of Ag-Ab molecules takes place leading to precipitation. For further elucidation of the mechanism of enzyme—antienzyme interaction it seemed helpful to compare the kinetics of the inhibition reaction and the precipitin reaction.

For these experiments the concentrations of Ag and Ab were adjusted to allow enzymatic and turbidimetric measurements simultaneously. The Ab content of the gamma globulin fraction was estimated with the quantitative precipitin test using a highly purified YADH preparation which was uniform in electrophoretic analysis. The equivalence zone was characterized by a molar Ag: Ab ratio of 1:3.

Enzymatic and turbidimetric measurements were carried out under identical conditions in phosphate buffer 0.067 *M*, pH 7.4

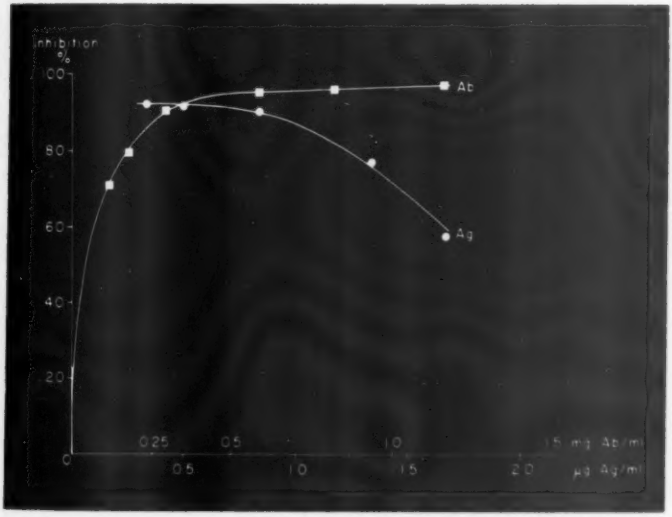

FIG. 3. Influence of Ag and Ab concentration on the maximal inhibition. Buffer, DPN, and substrate concentrations as in previous experiments; Ag concentration (in experiments with varying Ab concentration) 1.22µg YADH/ml, Ab concentration (in experiments with increasing Ag concentration) 30 µg gamma globulin.

with the exception that ethanol was omitted from the test solution for turbidity recordings. DPN concentration 0.0012 *M*, Ab 1.3 mg precipitable protein per ml (estimated by biuret reaction) YADH 1.73 and 2.46 μg protein/ml respectively. The inhibition reaction was measured at 366 mμ in cells with a light path of 0.5 cm, total volume 1.00 ml. Reaction rates were obtained with the zero-time method for the first minute and preincubation method for the following measurements. Only in the experiments with the doubled Ag concentration a slight and increasing interference due to light scattering was observed after about 10 minutes of reaction time; the corrections necessary to eliminate this effect were however minimal.

Fig. 4 demonstrates the results of enzymatic and turbidimetric measurements of Ag-Ab interaction. The kinetics of the inhibition reaction and the precipitin reaction are distinctly different in character. The inhibition of the enzymatic activity by the

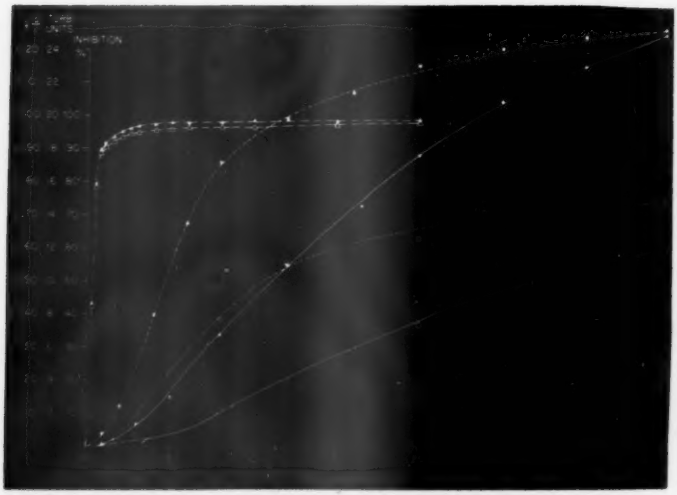

FIG. 4. Comparison of turbidimetric and enzymatic measurements of Ag Ab reaction: Ab concentration 1.3 mg/ml, YADH 1.73 and 2.46 μg/ml.

- transmittancy measurement (1.73 μg YADH)
- transmittancy measurement (2.46 μg YADH)
- nephelometric measurements (1.73 μg YADH)
- nephelometric measurement ,2.46 μg YADH)
- enzymatic measurement (1.73 μg YADH)
- enzymatic measurement (2.46 μg YADH)

Ab reflects an early phase in the formation of Ag-Ab complex. Maximal inhibition, within this range of concentrations, is obtained in three minutes while the turbidity curves represent slower functions. The aggregation and precipitation measurable with the nephelometric and transmittancy method proceed over a longer time, reaching an apparent endpoint at about 20 to 80 minutes (according to Ag:Ab ratios) but continuing to a slight degree for about 24 hours. The nephelometric curves differ from the transmittancy curves insofar as they show a more rapid increase at the starting phase of the reaction. Both methods provide, however, only relative data since the concomitant increase in particle number and particle size, taking place during the Ag-Ab reaction, leads to changes in light absorption as well as light scattering.

Doubling of the Ag concentration increases the rate and the extent of the turbidity reaction but is without influence on the degree of inhibition. This is explained by the saturation of the enzyme with inhibitory Ab in the zone of Ab excess, resulting in an almost 100% loss of activity. Increase in the Ag amount is ineffective as long as concentrations remain in the asymptotic range of the inhibition curve (see Fig. 3).

Influence of hydrogen ion concentration on reaction rate of Ag-Ab interaction. The investigation of relationships between concentration of hydrogen ions and the kinetics of the YADH-Ab reaction offered another possibility for the comparison of enzymatic and turbidimetric measurement. The experiments were designed to reflect the rate of reaction in the early phase of the Ag-Ab combination in which overlapping of both functions was shown to exist. The ionic strength of the buffer was reduced to 0.10 and kept constant over the range of change in pH. Concentration of Ab was 1.4 mg/ml, YADH 3.2 μg/ml, DPN and ethanol as in previous experiments (the latter was omitted in measurement of turbidity). The hydrogen ion concentration was varied from pH 5.9 to 8.6. Rapid inactivation and precipitation of the enzyme (isoelectric point) occurred in attempts to extend the studies into lower pH values. Fig. 5 indicates the effects of changes in pH on the inhibition rate and precipitin reaction.

The turbidimetric data, gained by transmission measurement at 366 mμ, light path 4 cm, are expressed as increase in optical density in the time interval from 1 to 3 minutes after start of the

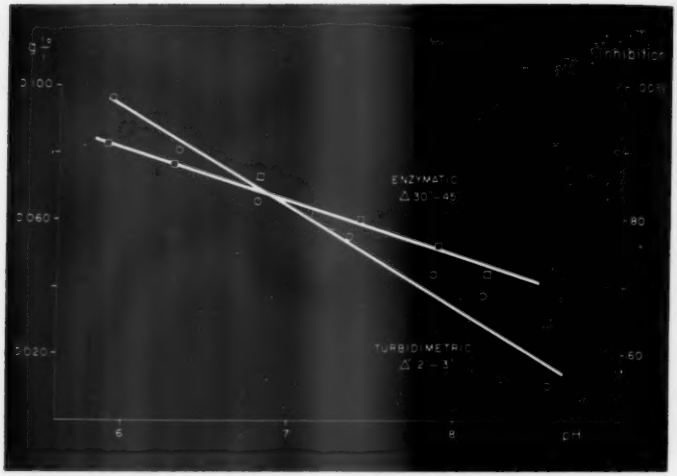

FIG. 5. Influence of hydrogen ion concentration on reaction rates of enzyme-antienzyme interaction:

o———————o turbidimetric measurement ($\log \frac{i_o}{i}$)

□———————□ enzymatic measurement (inhibition in per cent)

For the description of the experiment see text.

reaction. The enzymatic values represent inhibition in per cent, calculated from corresponding activities at time interval 30–45 seconds of tests with Ab and without Ab respectively.

Within the range of pH 5.9 to 8.6, an almost linear decrease in reaction rates was observed with both methods. The turbidity dropped from an optical density of 0.082 at the acid side to 0.010 in the basic milieu while the corresponding changes in inhibition were from 91% at pH 5.9 to 72% at pH 8.2. The optimal pH of the YADH activity was found to be around 7.9 with a rapid decline of the rate of reaction towards lower pH. It was anticipated that the calculation of the rate of inhibition in per cent of blank activity at corresponding pH would reflect the direct influence of pH on the Ag-Ab interaction, eliminating the pH dependence of the enzyme. From the results, that reaction rates of the turbidimetric and inhibition measurements decrease with increasing pH, the conclusion can be drawn that in this early phase of the Ag-Ab reaction both functions (inactivation and precipitation)

require the dissociation of basic groups either at the Ab or at the Ag molecule.

Discussion

The reason that enzyme-Ab studies have found special consideration in immunological research (2) seems to be more a matter of methodology than of basic nature. The involvement of the catalytic activity of the Ag molecule in the Ag-Ab interaction is a rather fortunate occurrence but is not a requisite since systems have been described in which precipitation was observed without concomitant loss of enzyme activity (1, 6).

The advantages for the use of YADH as Ag can be summarized in the following points: the enzyme is easily obtained in pure form (10), its properties are well defined (13, 12), it possesses good antigenicity, and the spectrophotometric measurement of the enzyme activity makes direct recordings of reaction rates possible. The Ab's produced against the yeast enzyme do not cross react with horse liver alcohol dehydrogenase (LADH) as might be expected, considering the difference in the origin of these two proteins (5); but the finding is of interest from the enzymological point of view, because both enzymes are DPN-linked, have quite similar substrate specificities, require free SH-groups for their catalytic activity, and contain zinc atoms in their molecules.

The results gained with enzyme-Ab studies depend on the technical approach and the mechanism which causes the inactivation. By analyzing the YADH system a difference between the zero-time method and preincubation method was detected which did not reflect altered conditions in the Ag-Ab combination but could be explained as an effect of the formation of an enzyme-DPNH complex. Both methods can be expected to give identical results when the enzyme reaction follows a first order function and differences have to be anticipated in enzyme systems in which accumulation of a reaction product exerts an inhibitory action on the reaction velocity.

In the YADH-rabbit Ab system, formation of the Ag-Ab complex does not involve competition for the same binding sites of the Ag of either substrate and Ab or coenzyme and Ab. The extent and the rate of the reaction are not altered by the sequence in which the different components of the enzymologic and im-

munologic systems are added. Competitive mechanisms have been described, however, in other enzyme-antienzyme studies (7, 4).

Zinc bonds and —SH groups of YADH probably do not participate in the Ag-Ab interaction. Experiments in which the Ag was pretreated with *o*-phenanthroline (in concentrations sufficient to chelate about 50% of the Zn bonds) and titrations of the free —SH groups with *p*-chloromercuribenzoate were, however, not conclusive because of difficulties in stabilizing the enzyme activity.

The inhibition reaction can be measured in Ab excess only. The rate of Ag-Ab combination increases with increasing Ab concentration. The complex is soluble and dissociable during the early phase of the reaction. The demonstration of two "stages" by simultaneous measurement of enzyme inhibition and precipitation does not imply that these reactions proceed independently. Since the early inhibition phase is a reversible reaction, the following aggregation and precipitation can be expected to change the equilibrium by the formation of "irreversible" and insoluble enzyme-Ab complexes. The similarity of the influence of hydrogen ion concentration on the reaction rates would indicate such a relationship since in both systems the rates increased with decreasing pH. An interdependence of the two reactions could be assumed also in view of a report of McBroom Junge, *et al.* (8). These authors found, analyzing a yeast glyceraldehyde 3-phosphate dehydrogenase—rabbit antiserum system with turbidity measurements (transmission measurement of scattered light), that in Ab excess the part of the reaction which is rate-determining is a second-order reaction or a sum or sequence of second-order reactions.

It is unlikely that the mechanism of YADH-Ab interaction includes competition for the active sites or other essential groups of the enzyme. The most probable explanation of the inhibition effect would then be that the Ab attaches itself to special configurations of the enzyme molecule and by covering the active site (or sites) creates a steric hindrance which prevents the free access of substrate to the enzyme.

Enzyme-antienzyme systems offer a special advantage for *in vitro* studies of basic factors and kinetics of Ag-Ab interactions. The difficulties in integrating the results of different studies are

mainly due to the technical approach (sequence of addition of components, use of preincubation method or zero-time method), apparent chemical diversity of Ab's formed against the enzyme and involvement of different mechanisms of interaction.

Summary

The yeast alcohol dehydrogenase-rabbit antiserum system is analyzed with enzymological and turbidimetric methods. The inhibition reaction represents an early phase of the formation of antigen-antibody complex which can be separated from the slower proceeding precipitin reaction. The reaction rates of both phases are similarly influenced by the hydrogen ion concentration indicating the importance of the dissociation of basic groups involved in the antigen-antibody reaction.

Different methodical arrangements are used to investigate the mechanism of enzyme-antibody interaction. The results render it unlikely that competitive mechanisms exist for the same binding site of the enzyme between antibody and substrate or antibody and coenzyme. Other possibilities of interference of the antibody with the enzyme activity are discussed.

Acknowledgment

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PREPUTIAL GLANDS OF THE RAT AS AFFECTED BY PITUITARY HORMONES[a]

EDWARD G. RENNELS, DOROTHY M. ANIGSTEIN AND LUDWIK ANIGSTEIN[b]

Introduction

The size of the preputial glands in the rat is apparently influenced by a number of steroid hormones and also by hormones of the anterior pituitary gland. The early studies of Korenchevsky (see review, 1944) established that a number of androgens are capable of stimulating these glands. That such action is independent of the pituitary has been shown in extensive studies by Huggins *et al.* (1955) in hypophysectomized animals. It was also shown that extracts of the anterior lobe which were high in ACTH activity were effective in increasing the weight of these glands in the rat (Noble and Collip, 1941 a, b). Several publications have since appeared which seem to support the view that ACTH may exert a direct stimulating action on these glands. Thus, Jacot and Selye (1951 and 1952) showed that ACTH preparations could cause a significant enlargement of these organs in female rats which were both adrenalectomized and ovariectomized. Unfortunately these animals were not hypophysectomized, so it seems possible that endogenous ACTH and other pituitary hormones may have contributed to the observed effect. Some effect of ACTH in preventing the post-hypophysectomy atrophy of preputials has been reported by Hess *et al.* (1953). It was also reported that adrenalectomy for 3 weeks led to preputial gland hypertrophy which could be partially prevented by desoxycorticosterone acetate (DCA) (Hess *et al.* 1953) and that the intravenous injection of ACTH would produce a significant fall in the ascorbic acid of the preputial glands of hypophysectomized rats (Hess *et al.* 1952). Finally, a marked hypertrophy of the

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preputial glands in animals subjected to chronic electro-shock stress has been reported recently by Hall and Hall (1959). There seems little doubt, therefore, that stress in the intact rat or ACTH in the hypophysectomized rat can act to stimulate preputial glands. It is not yet clear, however, to what extent such stimulation is due to a direct effect of ACTH, as contrasted with an adrenal mediated response, or to the combined effects of ACTH and other pituitary hormones. The present paper will attempt to clarify this point.

In addition to ACTH, other anterior lobe hormones may affect the size of the preputial glands. Thus, growth hormone has been found to act synergistically with testosterone in stimulating the preputial glands of hypophysectomized rats (Huggins *et al.* 1955). It is not yet clear whether this represents anything more than an expression of the general anabolic effects of growth hormone. Of the other pituitary hormones only luteotrophin seems capable of stimulating preputial glands in the absence of the pituitary and gonads (Callahan, 1958). Such stimulation has been obtained by endogenous luteotrophin as secreted by pituitary grafts (Sanders and Rennels, 1959) as well as by the injection of purified preparations (Callahan 1959). This growth was attributed to a direct effect of luteotrophin on preputial glands in contrast to the marked activity of the other gonadotrophins (FSH and LH) which is dependent on their stimulating androgen secretion.

It would appear, therefore, that the observation of preputial gland hypertrophy in rats must be interpreted very cautiously until more details are known of the way in which these glands respond to hormones of the pituitary, adrenals and gonads, both singly and in combination. It is hoped that the present communication will contribute to our understanding of this problem.

Materials and Methods

Data from two otherwise unrelated studies are included since they both contribute information relative to the problem. In the first study hypophysectomized female Holtzman rats were treated daily with pituitary hormones for a period of six weeks. These animals were hypophysectomized at 46 days of age by the usual parapharyngeal approach using ether anesthesia. In one group of animals the ovaries and adrenals were removed at the time of hypophysectomy. Hormone injections were begun on the day following the operations, and throughout the experiment the animals were kept in air-conditioned quarters. Five per cent sucrose was given the animals to drink and a supple-

ment of lettuce and carrots added to the regular diet of Purina laboratory chow. At the termination of the experiment the animals were killed by decapitation. A careful check of the sellar region was made for remnants of pituitary tissue. The following organs were cleaned and weighed on a torsion balance: ovaries, and adrenals (when present), thymus, thyroid, uterus, vagina and preputial glands. This information, together with the body weight records and observations of hair texture (Rennels and Callahan, 1959) served as criteria for determining the completeness of the hypophysectomies. All animals included in this series were judged to be completely hypophysectomized. The animal groups and hormone treatments given them are indicated in Table 1.

In the second study Holtzman female rats were prepared by hypophysectomy at an age of 28 days for the standard tibial test for growth hormone (GH). Two weeks later injections of GH or rat pituitary homogenate were made over a four day period and on the fifth day the animals were autopsied. At this time both tibiae were dissected and split and a number of organs reflecting pituitary function were cleaned and weighed on a torsion balance. This study was designed to measure the inhibitory capacity of antisera against rat pituitary homogenate and against purified growth hormone preparations and the results of this aspect of these experiments have been reported (Anigstein, *et al.* 1960). Since preputial glands were weighed routinely it was of interest to compare the effects produced by rat pituitary homogenate and purified growth hormone on these glands. The dosage of pituitary homogenate given over the four day period was sufficient to produce a marked stimulation of body growth, tibial plate width and ovarian and uterine weights. For the present considerations it is sufficient to present only the data

TABLE 1

Effect of Hormone Treatment on Preputial Glands of Hypophysectomized Female Rats

Animal Group	No.	Hormone Treatment	Final Body Wt. (g.)	Adrenal Glands (mg.)	Preputial Glands (mg.)	(mg./100 gm.)
Hypex.	9	None	135 ± 6.0*	13.8 ± 0.6	27.7	20.5 ± 0.9
Hypex.	5	4 I.U. ACTH/day[a]	121 ± 3.0	51.7 ± 2.5	83.1	69.0 ± 6.4
Hypex.	5	40 I.U. LTH/day[b]	145 ± 4.1	11.5 ± 0.6	42.4	29.3 ± 1.7
Hypex.	5	1 mgm. GH/day[c]	205 ± 7.5	15.9 ± 0.9	39.4	19.3 ± 1.3
Hypex. Ovx. Adx.	5	4 I.U. ACTH/day[a]	141 ± 4.0		33.7	23.9 ± 1.7

* Standard error.
a Wilson (ACTH) Corticotropin Solution.
b Armour Panlitar Lactogenic Hormone. This preparation was a gift from the Endocrinology Study Section, National Institutes of Health.
c Armour Growth Hormone, lot no R285128.

relative to preputial gland and body weight in the several groups of assay animals. These data are included in Table 2.

Results and Discussion

As shown in Table 1, ACTH, when given to hypophysectomized animals over a period of 6 weeks, will produce a remarkable hypertrophy of the preputial glands. This effect is almost completely lost, however if the animals are surgically deprived of their adrenals and gonads. In the latter case only a very insignificant increase in preputial gland weight occurs (23.9 compared to 20.5 mg/100 g). As anticipated, this prolonged ACTH treatment produced a considerable enlargement of the adrenals and it is probable that adrenal androgen secretion was largely responsible for the stimulation of the preputial glands since C_{21} corticoids are inactive in this respect in hypophysectomized rats (Huggins *et al.* 1955). Also, it was noted that the ACTH injections were completely without effect on ovarian weight or histology indicating that the gonads are not involved.

Evidence that luteotrophin is capable of stimulating preputial and sebaceous glands in hypophysectomized rats was obtained recently by Callahan (1958, 1959). The data in Table 1 confirms

TABLE 2

*Effect of Rat Pituitary Homogenate or Bovine Growth Hormone on Preputial Glands in Hypophysectomized Rats***

Material Injected	No.	Final Body Wt. (g.)	Preputial Glands (mg./100 gm.)
None	10	76±2.4*	20.6±1.4
Normal Rabbit Serum[a]	15	79±1.4	20.3±1.0
Rat Pituitary Homogenate[b]	23	91±1.2	33.9±1.1
Bovine GH (Armour)[c]			
100 μg	6	87±1.5	21.1±1.0
400 μg	6	88±1.9	22.5±2.0
Bovine GH (Li)[d]			
48 μg	17	82±1.0	22.6±1.0
96 μg	5	89±2.1	22.2±4.0

* Standard error.

** All rats were hypophysectomized at 28 days of age. Injections were made over a 4 day period beginning 2 weeks post-operatively.

a Dosage: 0.5 ml. intraperitoneally.

b Dosage: 0.41 mg. rat pituitary homogenate per rat, subcutaneously 2x daily for 4 days. Total: 3.3 mg./rat.

c Growth Hormone, (Somar) supplied by Armour and Company.

d This preparation of highly purified bovine growth hormone was kindly supplied by Professor C. H. Li of the Hormone Research Laboratory, Berkeley, California.

this finding since this treatment produced a highly significant increase in the weight of the preputial glands ($P = < .001$) as compared to the untreated controls. This would seem to be a direct effect of luteotrophin (prolactin) since no stimulation of adrenals or ovaries was observed in these animals. Such an action for luteotrophin is not surprising in view of the role it has been shown to play in the maturation of hair in the rat (Rennels and Callahan, 1959).

The growth hormone used in this experiment was devoid of any specific stimulating effect on the preputial glands although it was effective in promoting a near normal growth rate of the animals. Although it has been shown to act synergistically with testosterone on the preputial glands of hypophysectomized rats (Huggins *et al.* 1955) growth hormone would seem to have no independent action in this regard.

The results of the second study (Table 2) confirm the failure of growth hormone to influence preputial growth in hypophysectomized rats. The dose of the two GH preparations given was much smaller than in the other study and the injection period only four days. Still, this was sufficient time to effect a highly significant ($P = < .001$) increase in preputial gland weight by the injection of rat pituitary homogenate. The larger dose of both GH preparations produced increases in body weight and tibial plate widths which were only slightly less than that produced by the rat pituitary homogenate. The results shown in Table 2 emphasize the potent stimulatory effect which the whole complex of pituitary hormones can exert in a very brief time period in an animal deprived of its own pituitary gland.

We may ask then to what extent the pituitary hormones are normally involved in controlling the size of the preputial glands? Certainly we must consider the gonadotrophins (FSH and LH) of primary importance in the male through their role in promoting androgen secretion by the Leydig cells. In the female we do not know the extent to which ovarian androgens are normally secreted. Likewise, the pituitary stimulus for and extent of adrenal androgen secretion under normal and abnormal conditions is almost entirely unknown. Next in importance in terms of the magnitude of the preputial response is ACTH. In the presence of the adrenal glands this hormone can produce a remarkable hypertrophy of preputial glands. Does it have any direct effect on the

size or metabolism of these glands? This question has apparently not been studied in hypophysectomized-adrenalectomized animals before and it was noted that under these conditions ACTH produced no marked effect on the weight of the preputial glands. Statistically, the modest increase observed (23.9 compared to 20.5 mg/100 g) is significant at between the .05 and .10 level of probability so that some very minor direct action of ACTH seems possible. The degree of hypertrophy reported by Jacot and Selye (1951, 1952) in adrenalectomized-gonadectomized rats was likewise moderate even though they found a value of P which was less than .01. In their experiments one must acknowledge the presence of increased endogenous ACTH and the fact that the dosage of ACTH given was of pharmacologic proportions. Also, the possibility of luteotrophin secretion from the pituitary gland being responsible in part for the hypertrophy they observed must now be given consideration. An attempt was made by Jacot and Selye to rule out luteotrophin involvement by injections of prolactin but the dosage used in this case was too minute to have possibly caused any effect on the animals (.17 IU per day). In the experiments of Hess *et al.* (1952) in which adrenalectomy was found to increase preputial gland weight it can also be stated that a relatively minor enlargement of the preputial glands was found. Again the experiment was inconclusive in regard to the direct action of ACTH due to the unknown contribution made by the animals' pituitary in regards to gonadotrophin and especially luteotrophin secretion. From the results of the present study and those of Callahan (1959) there can be no doubt that luteotrophin is of real importance in the control of preputial gland size. Unfortunately, the extent to which this hormone may be released under normal and stressful conditions has not been studied in any quantitative manner. Consequently, in view of the several hormonal factors which may modify preputial gland size it would seem unwise at present to attribute the enlargement of these glands to any single or specific alteration in the hormonal milieu. Such enlargement however, may serve as a useful index of increased pituitary and androgenic hormone levels and in this way provide another means of assessing the amount of stress various experimental procedures produce. This would seem to be the case in the observation of preputial gland hypertrophy induced by

chronic electro-shock in the recent experiments of Hall and Hall (1959).

Summary

Earlier work has shown that the preputial glands of the rat respond by hypertrophy to a number of steroid hormones and also to certain pituitary hormones. The response of these glands to injections of ACTH, growth hormone and luteotrophin has been investigated in hypophysectomized, female rats. In one study, in which daily injections were given over a 6-week period, it was found that growth hormone was without any specific stimulatory effect on the preputial glands while both ACTH and luteotrophin caused a highly significant increase in the weight of these glands. Whereas the effect of ACTH was almost entirely dependent on the presence of the adrenal glands, stimulation by luteotrophin occurred in the absence of any increase in adrenal weight. In a second study daily injections of rat pituitary homogenate or purified growth hormone were given hypophysectomized rats for a 4-day period. The pituitary homogenate produced a marked increase in preputial gland weight but the purified growth hormone was without effect.

Consideration is given to the role played by the several pituitary hormones in controlling preputial gland size. It is concluded that preputial gland hypertrophy may serve as a useful index of increased pituitary and androgenic hormone levels in the rat.

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MUSCULAR DYSTROPHY: SPONTANEOUS OCCURRENCE IN DUCKS[a]

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Necrosis of muscle and weakness have been observed in the fish, chicken, duck, lamb, calf, foal, tree kangaroo, mouse, rat, cotton rat, hamster, guinea pig, rabbit, dog, and monkey (12). The etiology of the necrosis frequently is unknown; however, it may result from a deficiency of vitamins (12, 15, 16, 10, and 11), potassium (20, 3), choline (9), in cortisone-treated rabbits (6) and in bacterial infections (23, 7) and viral (4, 8). Muscular dystrophy in man and the mouse may be hereditary (22, 2, 13).

In Symposium on Nutrition (21) numerous observations have been recorded on the clinical, pathologic and biochemical characteristics of muscular dystrophy. Milhorat (14) has said that progressive muscular dystrophy in man resembles, in practically all of its essential features, the muscular dystrophy produced experimentally in several species of animals by dietary deprivation of vitamin E. Contractures, which often are of great importance in the development of disability in progressive muscular dystrophy, are usually considered as arising subsequent to muscular wasting and weakness due to an impairment in a metabolic mechanism.

The white Pekin duck has been used in our laboratory for many years in the experimental study of malaria, vitamin A deficiency, and more recently carcinogenesis. For many years we have observed the presence of one or two ducklings, within a group of 25 to 50 birds, with abnormalities of either one or both legs. This abnormality usually occurred in birds three to six weeks of age. Because of the well known, but uncomplimentary, remarks referable to the "locomotion" of the duck, we always considered such abnormalities as "congenital" in origin.

Recently focal areas of necrosis have been observed in the

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striated muscles of turkeys in our laboratory (18, 19). Some of these birds developed difficulty in walking. Histologic study of the striated muscles in the white Pekin duck has shown focal areas of Zenker-like degeneration involving individual muscle fibers. This lesion has been observed now in approximately 100 ducks. It has been found rarely in birds less than two weeks of age. The association of spontaneous-occurring muscular necrosis, with weakness and abnormalities of the extremities, in ducks is the basis of this report.

Four ducks with abnormalities of the legs have been selected to illustrate the clinical and pathologic changes. A detailed study of the age and frequency of occurrence of muscular necrosis and observations on the etiology will be the basis of a subsequent study.

Methods and Materials

One-day old white Pekin ducks were obtained in groups of 25 and 50 from a commercial hatchery. They were kept in batteries with food and water available at all times. Purina Duck Startena and Growena* were fed. Birds at varying ages were sacrified by severing the cervical portion of the spinal cord. A complete examination usually was made, including sections from the viscera, brain, spinal cord, peripheral nerves and muscles. The tissues were fixed in a four per cent solution of formaldehyde. Paraffin sections were prepared and stained routinely with hematoxylin and eosin. Select sections were stained with periodic acid Schiff's reagent, Gomori's methenamine silver and Heidenhain aniline blue.

Photographs and roentgenograms were made of some of these birds.

Results

Case 1. Duck 1810 was selected to illustrate the pathologic change in the muscles. This bird was received on December 17, 1959. On the 27th day the left leg was noticed for the first time to be held in extension (Fig. 1). The right leg was normal. Other than for the extension of the left leg, this duck looked like all others. It was sacrificed on the 28th day. No gross lesions were observed. A microscopic area of acute necrosis was present in one of the three sections of pectoral muscle. Necrosis was present histologically in two of the six sections of muscle from the left leg. Lesions were observed in three of the four sections of muscle removed from the right leg.

* Ralston Purina Co., St. Louis, Mo.

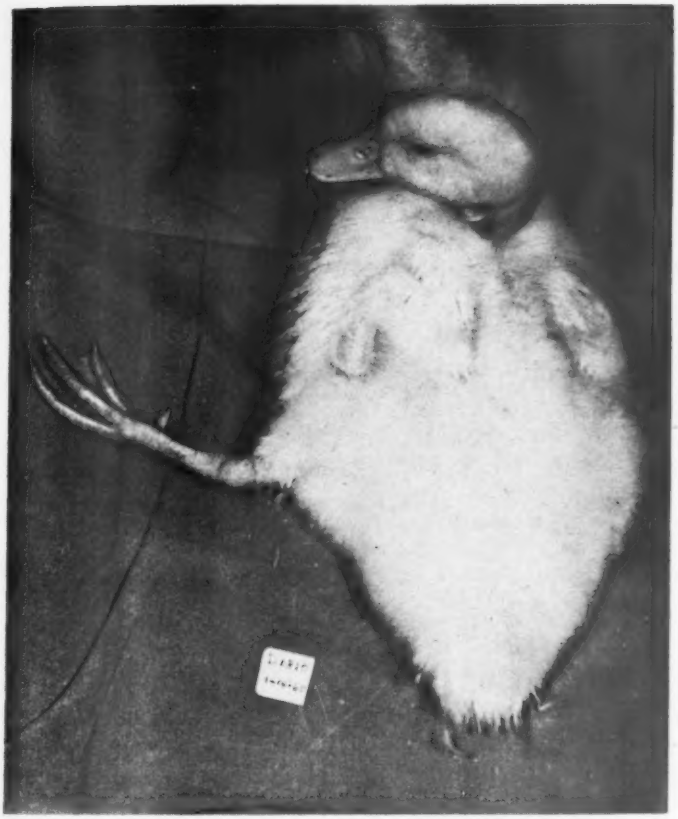

FIG. 1. Duck 1810. The left leg is held in extension. This was first observed when the duck was 27 days of age. Photographed on the 27th day. Histologic changes in the muscles of the left leg are shown in FIGS. 2, 3, 4, and 5.

The necrosis in the striated muscle involved small segments of individual fibers (see illustration in Case 2). The earliest change observed was swelling and loss of striations. Sometimes these areas of necrosis were three or four times the normal width of a muscle fiber and stained much deeper with eosin than the normal muscle. Usually there was a sharp line of demarcation between the swollen, eosinophilic-staining, degenerating portion of muscle fiber and the uninvolved muscle. The area of junction between

the normal and necrotic muscle sometimes was narrow, granular and irregular, while other fibers showed a complete absence of the sarcoplasm with only a sarcolemmal tube filled with eosinophilic debris (Fig. 2). The degenerated portion of the muscle fiber often showed vacuoles of varying size. In the earlier stages of degeneration there was no accompanying cellular reaction. Leucocytes and mononuclear cells, however, subsequently infiltrated the necrotic area (Fig. 3). These cells were localized primarily within the sarcolemma. Small groups of lymphocytes frequently were present within a muscle.

Regeneration was difficult to follow in these sections of striated muscle. Sometimes a focal segment of a muscle fiber stained basophilic and had a proliferation of sarcolemmal nuclei (Fig. 4). Sometimes the degenerated portion of the muscle fiber was replaced by either fibroblasts or fat. No pathologic changes were observed in the pancreas, liver, kidney, spleen or intestines of this duck. No histologic observations were made on the nervous system.

Case 2. Duck 1838. This bird had bilateral weakness of the legs.

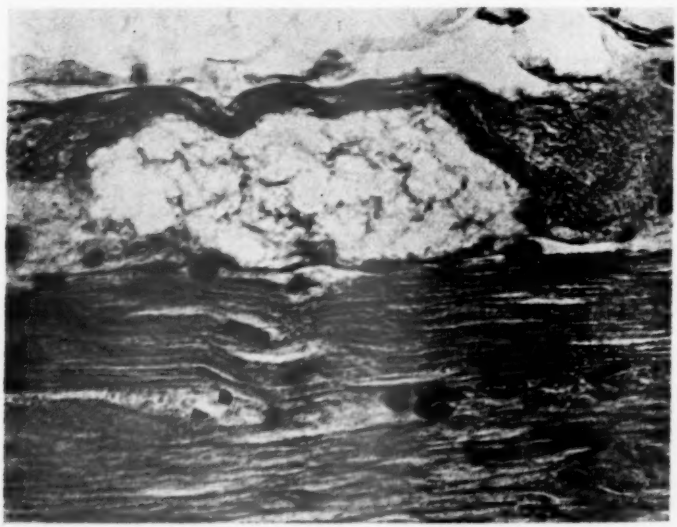

FIG. 2. Duck 1810. A focal area of the muscle fiber is completely degenerated leaving a sarcolemma-like tube. H&E × 712.

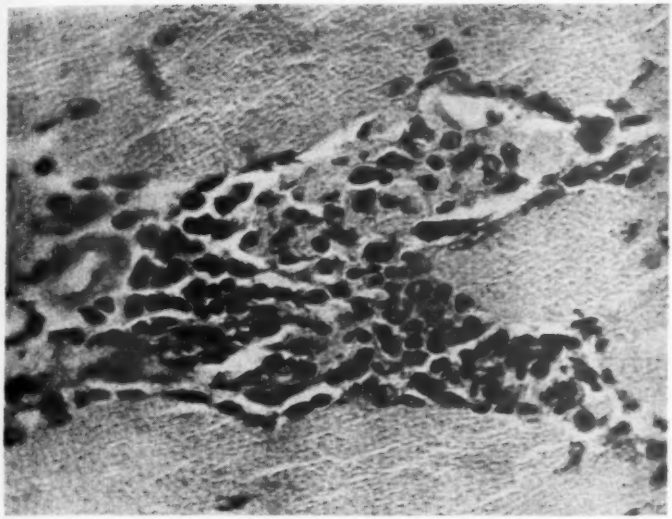

FIG. 3. Duck 1810. The degenerated areas in the muscle fibers sometimes are filled with leucocytes and mononuclear cells. H&E × 712.

It was received on January 13, 1960 and was one of a group of 50. On the 42nd day this duck was observed for the first time to have difficulty in walking. The weight at this time was 1230 grams, which was less than some of the other ducks in the group. A crust, approximately 1.5 × 1.0 cm., was present on the web of the right foot. Beneath this crust was a chronic superficial ulcer. Both legs were extended laterally and each foot was inverted. The legs were weak.

Duck 1838 was killed when 42 days of age. No gross lesions were observed in the viscera. In the microscopic study lesions were found in the spleen, femoral nerve and muscle. Multinucleated cells, consistent with giant cells, were distributed through the splenic pulp. In one section of the left femoral nerve there was a group of lymphocytes surrounding a small blood vessel. Although six sections were made from this nerve, only one group of lymphocytes was observed. Five sections were made from the muscles in the left leg and in two of these there were foci of necrosis similar to that described in Case 1 (Fig. 5). No areas of

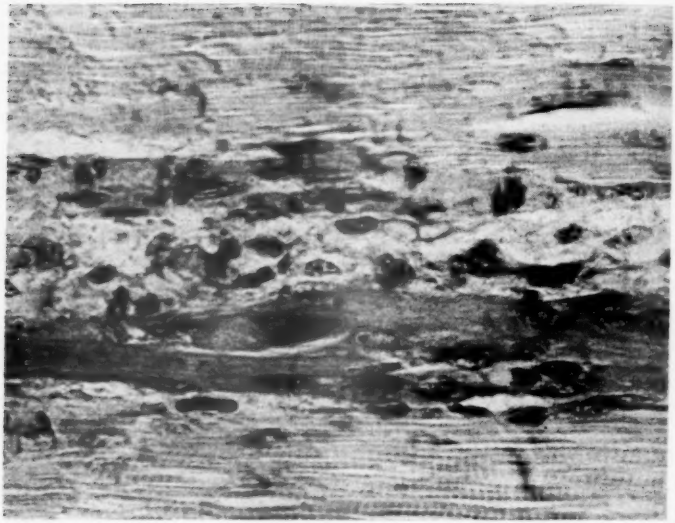

FIG. 4. Duck 1810. The degenerated areas in the muscle fibers sometimes are filled with basophilic cytoplasm and sarcolemma-like nuclei. H&E × 712.

necrosis were found in six sections of muscle from the right leg. The right femoral nerve was normal.

Case 3. Duck 1839. This duck was received at the same time as duck 1838 shown in Case 2. The left foot was observed to be slightly inverted on the 42nd day. Two days later this duck was killed. The muscle volume of the left leg appeared to be less than that of the right. The bones in the left leg were slightly distorted; however, no pathologic changes were observed. The joints were grossly normal. Six sections were removed from the muscles in the right leg. In one of these were focal areas of acute necrosis similar to that shown in Figure 5. One histologic section showed some regenerated muscle fibers. There were no changes observed in the right femoral nerve.

Nine sections of muscle were studied from the left leg. Two of these had focal areas of necroses and in a third there were several areas of muscle regeneration. No changes were observed in the left femoral nerve. No changes were found in sections from the brain and spinal cord. No sections were removed from the viscera of this duck.

Case 4. Duck 1877. This duck is included to show the progression of clinical symptoms in this form of muscular dystrophy. Roentgenograms and a movie were made to illustrate the progression of this deformity and to show the disturbance in locomotion. This duck appeared to be normal when received on March 16, 1960. On the 15th day there was first noted a slight limp in the right leg. At this time the foot was inverted and there was a fine tremor. The left leg was normal. The inversion of the right foot progressively increased and this bird had progressively greater difficulty in walking (Fig. 6).

Duck 1877 did not gain in weight equal to that of normal ducks. On the 41st day this muscular dystrophic duck weighed 1750 grams and the control, 2190 grams. At this time there was definite restriction of movement in the right tibia-metatarsus joint. This joint was slightly larger than the left. Roentgenograms made on the 34th day showed a slight distortion of the bones about the ankle joint, but no specific lesions were present either within the bones or in the joint. Roentgenograms made on the 63rd day were similar to those made a month earlier. However, the latter

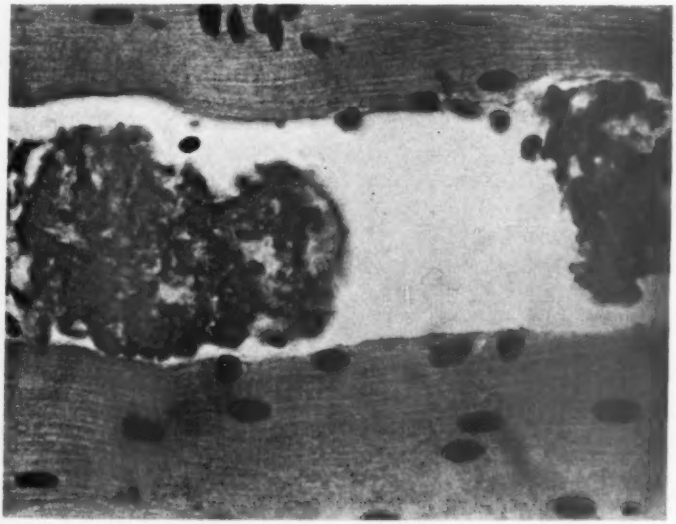

FIG. 5. Duck 1838. Acute necrosis in a muscle fiber on the 42nd day. Observe the absence of a cellular reaction. H&E × 712.

FIG. 6. Duck 1877. The right foot on the 34th day is inverted. This was first observed on the 15th day and progressively increased in severity.

films showed some coarse bony trabeculation in the tibia and metatarsus of both the right and left legs (Fig. 7).

This duck was killed when 78 days of age. The only pathologic changes found were in the striated muscles. These included sections from each leg and from the pectoral and cervical muscles. The lesions were similar to those observed in the other ducks in this study. It was of interest, however, to observe in this duck many regenerated muscle fibers and also focal areas in which portions of several muscle fibers had been replaced by fat. The overall histologic changes in this bird suggested an older process than was present in the ducks sacrificed at a younger age. No lesions were observed in several sections of brain and in one section of the femoral nerve removed from the right leg.

Discussion

The histopathologic changes present in the striated muscles of these ducks apparently are adequate to account for the abnormalities present in the extremities. As a result of the necroses in the muscles of the extremities, contractures occurred. The focal areas of degeneration in the striated muscles are not restricted to

those muscles of the extremities, but are present in the striated muscles throughout the body. The degree of involvement of the different muscles varies widely. Furthermore, in this study it is not possible to correlate the extent of the focal necroses in the muscles of an extremity with the gross abnormality.

The lesions in the striated muscles of these ducks are similar to the changes described in the muscles of chickens, ducks, guinea pigs and rabbits fed a vitamin E deficient ration (12, 11, 5, 17). They are also similar to the lesions described by Ellis (6) in cortisone-treated rabbits and by Dalldorf *et al.* (4) and Godman *et al.* (8) in virus-infected mice. The infiltration of fat between the muscle fibers and the fibrosis, as observed in duck 1877 (Case 4), is similar to the changes found by Brat *et al.* (1) in mice and by Brown (2) in a man 24 years of age with muscular dystrophy. Milhorat (14) pointed out the similarity of the lesions in man with progressive muscular dystrophy and those in animals deprived of vitamin E. We are unable to say at this time whether this degeneration in the muscles of these ducks is related to a deficiency of vitamin E.

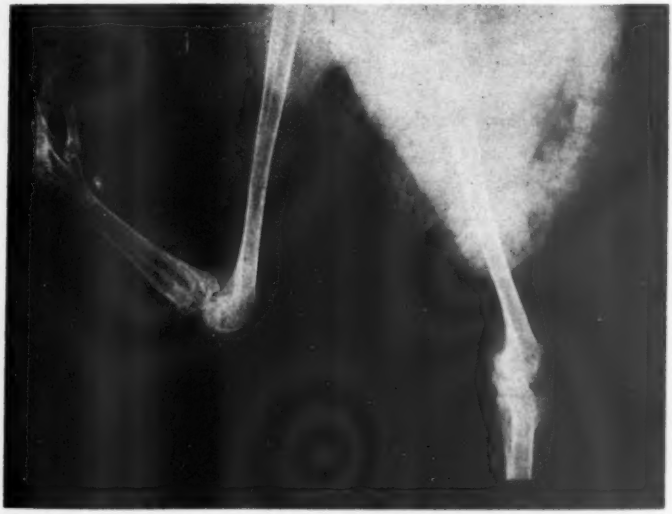

FIG. 7. Duck 1877. Roentgenogram of the duck shown in FIG. 9 made on the 63rd day. The right tibia-metatarsus joint is slightly enlarged. Bones in both extremities show coarse trabeculation.

The histologic changes in the muscles of the duck are the same as observed in turkeys with a pendulous crop (18, 19). A genetic factor apparently is not involved in this muscle degeneration in the duck and turkey since the ducks were obtained from a commercial hatchery and adult turkeys from different farms in several states. The wide distribution through this country of turkeys with this muscle necrosis would indicate that the etiology whatever it may be is not restricted to our laboratory. Studies are now in progress on the histology and the etiology of this form of muscular dystrophy in the duck and turkey. It occurs in a high percentage of the ducks that we have studied in our laboratory during the past several years.

Summary

The clinical and pathological changes observed in ducks with "malformed" extremities are reported. This abnormality is considered to be a contracture resulting from focal areas of necrosis in the striated muscles. This lesion occurs in striated muscles throughout the body and is not restricted to any specific anatomic site. It is a frequent lesion in our ducks. The etiology of this muscular dystrophy in the duck is under investigation but is unknown at this time.

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EFFECT OF RIBOFLAVIN-5-PHOSPHATE ON THE THROMBIN-INDUCED FIBRINOGEN-FIBRIN CONVERSION

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Thrombin occupies a key position in the coagulation of blood. Derived from its precursor, prothrombin, under the activating influence of thromboplastin, calcium, accelerin, convertin, and Stuart-Prower factor, this enzyme induces proteolytic cleavage of fibrinogen to form fibrin monomer and two fibrinopeptides. Active fibrin monomer then polymerizes to the characteristic proteinaceous gel which in blood is the fundamental matrix of the clot (7). A better understanding of this process, the final and crucial stage of blood coagulation, and of factors and conditions which may adversely affect it, is essential to future progress in diagnosis and treatment of hemorrhagic and thrombo-embolic diseases.

Thrombin has, in addition, esterase activity, although the physiologic significance of this property is not yet clear. Certain synthetic substrates, e.g., p-toluenesulfonylarginine methyl ester (TAMe) (8), are readily hydrolyzed, and for this reason have been employed frequently in assay of enzyme potency.

Landaburu and Seegers (3) have recently described conditions wherein thrombin's clotting activity is undeveloped (or lost) while its esterase potency is unaffected. On the basis of these observations, they have suggested that esterase thrombin may be the smallest (monomeric) degradation unit of prothrombin, while clotting thrombin may represent a dimeric form of the enzyme. This group has also noted esterase activity (but no clotting activity) in certain chemical modifications of thrombin (4).

Knowledge of the active sites of enzymes and the nature of their combination with substrates has often been enhanced by a

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study of appropriate inhibitors and antagonists. In the case of the thrombin-induced conversion of fibrinogen to fibrin, a variety of materials have been reported as inhibitors or retarders of enzyme activity. Of these, many are believed to act by blocking polymerization of the active fibrin monomer (9). However, to our knowledge selective inhibition of the clotting activity of thrombin is a rare finding. The literature affords evidence that other than Landaburu and Seegers (3, 4) only two investigators have reported such a selective inhibition of thrombin. Brambel observed that the anticoagulant, heparin, did not alter the esterase activity of thrombin (1). Similarly, Triantaphyllopoulos reported that this property was unaffected by the thrombin inhibiting Anticoagulant Fraction of Incubated Fibrinogen (A.F.I.F.) (11).

This paper presents evidence that selective inhibition of thrombin may be effected by riboflavin-5-phosphate (FMN), a discrete molecule of known structure. Furthermore, experiments with chemically related substances and inhibitor antagonists are described which provide information bearing on an understanding of the mechanism of inhibition.

Materials and Methods

Imidazole buffer, 1.72 g of imidazole (Eastman Kodak Co.) dissolved in 90 cc of 0.1N HCl, and diluted to 100 cc with distilled water, pH 7.2–7.4 (5).

Imidazole buffered saline, 95 parts 0.9 per cent saline buffered with 5 parts imidazole.

Fibrinogen, Bovine, (Armour and Co., Cohn Fraction I, 40–50 per cent citrate). This was dialyzed against imidazole buffered saline at room temperature, frozen at 2 per cent total protein concentration, and diluted with imidazole buffered saline to 0.5 per cent protein for clotting experiments.

Thrombin, Bovine, (Parke-Davis and Co., Topical). Approximately 5,000 N.I.H. units were dissolved in 10 cc 50 per cent glycerol, and stored at —20° C. This was diluted with imidazole buffered saline for clotting experiments.

Riboflavin-5-phosphate, monosodium salt (Nutritional Biochemicals Corp.). All concentrations were prepared in imidazole buffered saline.

L-cysteine hydrochloride (monohydrate), *Adenosine monophosphate* (muscle), *DL-alpha-tocopherol phosphate* (disodium salt), *Riboflavin*, (Nutritional Biochemicals Corp.). All these and following reagents were dissolved in imidazole buffered saline.

Glucose-1-phosphate (dipotassium salt), *Phenolphthalein disphosphate* (pentasodium salt hexahydrate), *Alpha-naphthyl phosphate* (monosodium salt), all purchased from Sigma Chemical Company.

Disodium phenyl phosphate, (Eastman Kodak Co.).

Protamine sulfate (Salmine), Eli Lilly and Co.).

L-Cystine, (Difco Laboratories, Inc.).

Thrombin Assays—Thrombin's clotting potency was measured as follows: To 0.4 cc of 0.5 per cent fibrinogen at 37°C was added 0.1 cc of thrombin. This latter solution was prepared from the stock thrombin described above by appropriate dilution with imidazole buffered saline, assuming the enzyme activity stipulated by the manufacturer, so as to yield 1 N.I.H. unit/0.1 cc. Under the conditions of this test, the clotting time of 16 ± 2 seconds was normally observed. A calibration curve was then prepared with serial dilutions of this thrombin solution. Clotting times were plotted against enzyme activity; this permitted the quantitation of residual thrombin potency in any unknown sample relative to the original or untreated specimen. Thrombin's esterase activity was assayed by the method of Sherry and Troll (8).

Inhibition Studies—Thrombin (or fibrinogen) was dissolved in imidazole buffered saline solutions of the specific inhibitors to yield the same final concentrations as used in the above clotting procedure, or esterase assay.

Results

Effect of FMN on thrombin. Thrombin, incubated at 37° C. with FMN of varying concentrations, showed rapid loss of clotting activity at concentrations greater than 10^{-2}M (Fig. 1). At lower FMN molarities, inhibition appeared to be slower and more time-dependent (Fig. 2).

Effect of FMN on fibrinogen. When fibrinogen was incubated with FMN as above, only the rapid, marked inhibition of more concentrated FMN was observed (Table 1).

TABLE 1

Thrombin-Fibrinogen clot times after incubation of substrate with varying concentrations of FMN

Incubation Time	Blank	10^{-2}M**	5×10^{-3}M	10^{-3}M	10^{-4}M	10^{-5}M
5 (Min.)	15.2*	Over 75	23.3	16.3	13.5	15.6
10 "	15.0	"	24.5	18.2	14.5	15.6
20 "	14.3	"	24.1	15.2	14.0	14.0
30 "	14.5	"	23.1	13.6	14.7	14.3
40 "	15.0	"	24.8	14.9	14.8	14.8
50 "	15.2	"	23.1	14.7	15.5	14.8
60 "	14.7	"	25.0	15.5	15.3	15.5
75 "	15.3	"	26.5	14.9	14.6	15.2

FMN is diluted $5 \times$ in order to assure the same inhibitor concentrations found in the clotting mixture with inhibited thrombin.

* Clot times in seconds.
** FMN Concentration.

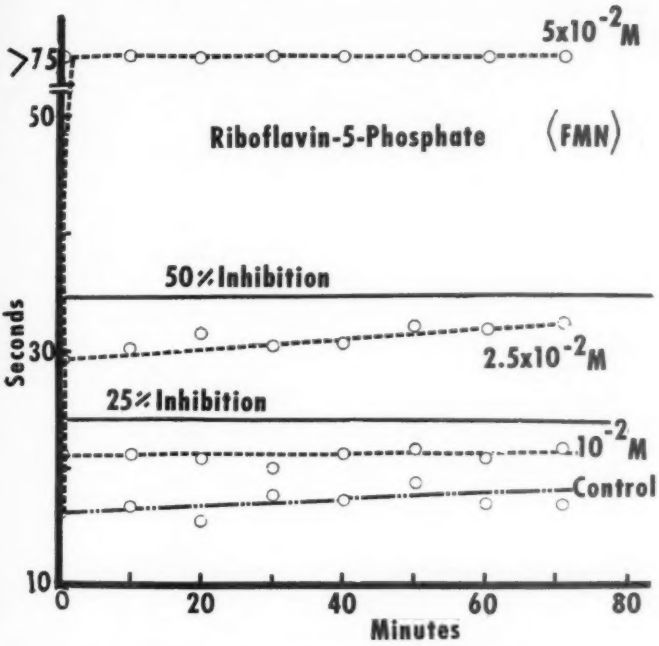

FIG. 1. Thrombin-Fibrinogen clotting times after incubation of enzyme with varying concentrations of FMN. Clotting times in seconds are plotted against incubation times in minutes.

Effect of riboflavin. Riboflavin at concentrations of 2.5×10^{-4}M (or less) had no demonstrable effect on clotting activity even after 75 minutes incubation with thrombin at 37° C. Higher concentrations of this material could not be tested because of its low solubility in aqueous media.

Effect of cationic materials. After thrombin had been inhibited by 75 minutes incubation with FMN, varying concentrations of ionic calcium (as calcium chloride solution) or of protamine sulfate were added in an effort to restore original activity. No restoration of enzymic activity was observed.

Effect of cysteine and cystine on FMN inhibited thrombin. After 75 minutes incubation of thrombin with 5×10^{-4}M FMN, varying concentrations of cysteine or of cystine were added. Incubation was continued at the same temperature for an additional

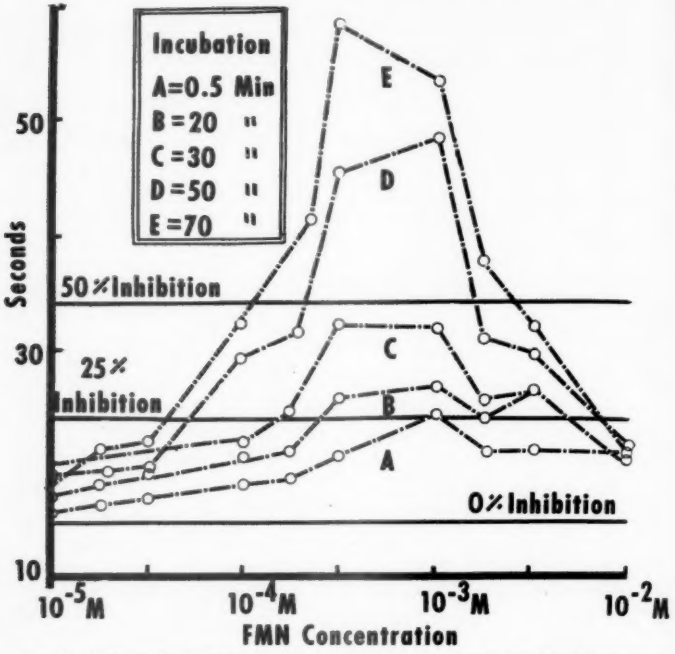

FIG. 2. Thrombin-Fibrinogen clotting times as a function of FMN concentrations and time of incubation with enzyme.

75 minutes. Partial, but not complete, restoration of clotting activity was achieved with each material (Table 2).

Effect of dialysis on FMN inhibited thrombin. Prolonged dialysis of FMN-inhibited thrombin against large volumes of distilled water at 4° C. eliminated all evidence of inhibition. Complete recovery of original activity was not achieved, but recovery of activity comparable to that of an uninhibited control specimen of thrombin submitted to the same dialysis conditions was achieved (FMN, previously mixed with fibrinogen, could also be removed in a comparable fashion so that no residual enzyme antagonism was demonstrable).

Treatment of thrombin with anions other than FMN. Various organic anionic materials, related to FMN, but differing in molecular dimensions, were incubated with thrombin under conditions described above (Fig. 3). It was observed that bulkier anions

TABLE 2

Incubation of cysteine or cystine with FMN inhibited thrombin. Control, uninhibited thrombin. Clot times in seconds.

Amino Acid Molarity	Cysteine FMN-thrombin	Control	Cystine FMN-thrombin	Control
1	Over 90	Over 90	..	..
10^{-1}	Over 90	Over 90	..	..
5×10^{-2}	80	25.8	47.4	21.5
2.5×10^{-2}	46.7	20.3	48.6	21.0
1.25×10^{-2}	..	..	50.4	23.0
10^{-2}	40.7	20.2	..	..
5×10^{-3}	50.5	18.8	Over 90	23.9
10^{-3}	43.6	25.8	..	..
5×10^{-4}	..	..	Over 90	23.8

produced rapid, marked inhibition of thrombin, characteristic of 5×10^{-2} M FMN; those of intermediate size showed predominantly slower; time-dependent inhibition seen with low concentrations of FMN; the smallest molecular sizes showed no inhibition.

Effect of FMN on thrombin esterase activity. Incubation of thrombin with FMN resulted in no significant loss of esterase activity. Variance in these experiments was such that very slight inhibition (less than 10%) would not have been detected although each result represents five-fold replication.

Discussion

These results indicate that FMN exerts a selective inhibitory action such that thrombin's clotting activity is masked while its esterase activity remains essentially intact. This would tend to substantiate from an entirely different experimental approach the interesting observations of Landaburu and Seegers concerning the divergence of proteolytic and esterase potencies of this enzyme, although not necessarily supporting their postulate as to the mechanism of such action. FMN would thus appear to mimic heparin in its selectivity.

At low FMN concentrations (10^{-5}M), no inhibition of thrombin is observed even after prolonged incubation. As FMN concentration increases, a time-dependent inhibition of thrombin occurs; this effect is most striking at FMN concentrations of 5×10^{-4}M

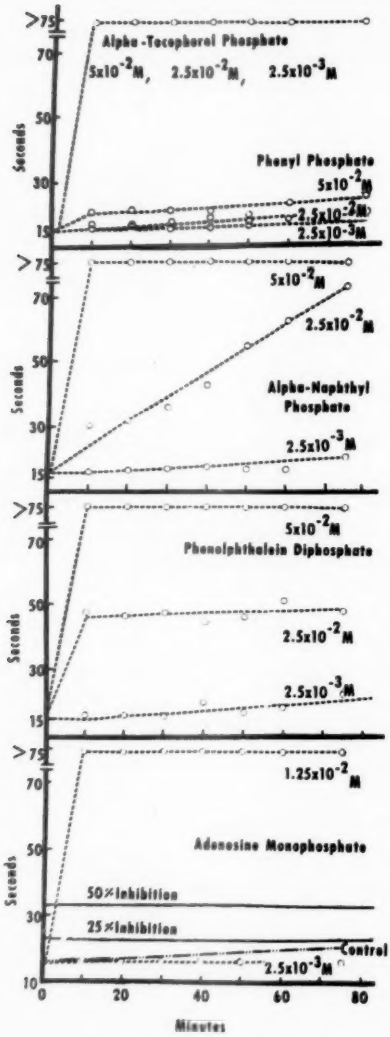

FIG. 3. Thrombin-Fibrinogen clotting times after incubation of enzyme with varying concentrations of various organic anions. Glucose-1-phosphate at concentrations of 5×10^{-2}M, 2.5×10^{-2}M and 2.5×10^{-3}M was also tested, however, it showed no inhibitory effect after 75 minutes incubation.

and 10^{-3}M. At higher concentrations (2.5×10^{-3}M to 10^{-2}M), the time-dependent inhibitory effect is diminished considerably. No fully adequate explanation for this phenomenon is apparent except the possibility of an association of FMN into a dimeric form, to cause an effective decrease in "active" FMN. Indeed, Michaelis and Schwarzenbach (6) observed that riboflavin associated into a dimeric complex consisting of one molecule of totally oxidized form and one of totally reduced form at pH 6.92, even at concentrations less than 5.4×10^{-4}M. At FMN concentrations greater than 10^{-2}M, rapid inactivation of thrombin and/or fibrinogen occurs. This rapid inactivation is probably due in part to increased ionic strength and lowered pH (0.31 and 6.3, respectively) of 5×10^{-2}M FMN. Shulman and Ferry (10) have observed that clotting time varies inversely with pH below 7.3 and directly with ionic strength. However, it should be noted that this rapid inactivation was not observed with glucose-1-phosphate at the same concentration, where ionic strength and pH should be comparable. Since at this high FMN concentration fibrinogen and/or thrombin are rapidly inactivated, it is impossible to ascertain the site of attack of FMN. However, the actual dimensions of FMN as a massive anion may play a significant role in the inactivation at this concentration which would not be observed with a smaller anion, such as glucose-1-phosphate.

Thrombin seemingly is more susceptible to FMN attack than fibrinogen, since clotting times increased progressively when FMN was incubated with thrombin, but remained essentially unchanged when the same concentrations of FMN were incubated with fibrinogen in a similar manner. Korsan-Bengtsen (2) observed a similar phenomenon using sodium laurate and sodium dodecylsulfonate, and suggested that thrombin has more cationic centers available at this pH than does fibrinogen.

The observations cited in this investigation strongly suggest that FMN exerts its inhibition by anionic attack on thrombin as the primary target. The fact that large organic anions, which are otherwise chemically unrelated to FMN, exert a similar antagonism, while anions of intermediate size are less effective and the smallest anions tested were ineffective, is most suggestive. One might picture the large organic half-ester of phosphoric acid as involved in ionic or hydrogen bonding with a basic group(s)

which is structurally near but not an essential part of the enzyme-combining site. By steric hindrance due to the molecular dimensions of FMN (and phenolphthalein diphosphate, adenosine monophosphate, alpha-tocopherol phosphate and naphthyl phosphate), the approach and attachment of the massive fibrinogen molecule might be blocked, while offering little hindrance to the much smaller substrate TAMe. On the basis of such a postulate, one presumes that the smaller anions (phenyl phosphate and glucose-1-phosphate) simply lack the size to provide this selective steric hindrance. This concept would not be incompatible with Seegers' view that acetylation of an essential amino group of thrombin has blocked clotting activity while retaining esterase activity, nor the view that heparin as a massive anion may act by a similar mechanism.

Unfortunately, the experiments with calcium, protamine, cysteine and cystine afford no conclusive proof of these views since only partial or no restoration of enzyme activity was achieved. For this reason, one cannot exclude the possibility that the time-dependent inhibition of thrombin may involve oxidative or reductive alterations of the thrombin molecule. However, the red-ox alteration of thrombin does seem unlikely in view of the apparent ease (by simple dialysis) of restoration of biologic activity. This suggests hydrogen or ionic bonding rather than alterations in primary covalent bonds. This general conclusion is strengthened by the finding that riboflavin, which has all the multifunctional potentialities of FMN except anionic character, is inactive under identical conditions.

Summary

Thrombin and/or fibrinogen was treated with riboflavin-5-phosphate (FMN). An anticoagulant effect was observed at high FMN concentrations upon both enzyme and substrate, and upon enzyme only, at low FMN concentrations. The FMN inhibited thrombin exhibited normal esterase activity. Various organic anions were also found to inhibit thrombin in a similar manner. Calcium and protamine sulfate were unable to reverse FMN's effect on thrombin, while cysteine and cystine were partially effective. Dialysis reversed the FMN inhibition phenomenon. FMN is believed to act as a massive anion to produce selective steric hindrance effects.

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THE DEPLETION OF ASCORBIC ACID IN THE ADRENALS AND OTHER TISSUES OF THIAMINE DEFICIENT RATS[a]

DON W. STUBBS[b] AND KENNETH OTTIS

During the past three decades since ascorbic acid was first isolated from bovine adrenal glands (12), much has been written concerning the possible role of this vitamin in adrenal function. Despite considerable experimentation and speculation, the high concentration of ascorbic acid in the normal adrenal and its rapid depletion following stress still pose obscure significance. As it has been shown that little, if any, synthesis of ascorbic acid occurs in the adrenal (9, 3, 7), the relationship between the adrenal stores of this vitamin and those of other tissues assumes greater importance.

Considerable evidence has been presented that the synthesis and storage of ascorbic acid is in some way dependent upon the availability of several of the B vitamins (4, 8, 10, 13, 2, 5). However, none of these previous investigators have followed the effects of the deficiency diets on ascorbic acid concentrations of various tissues, chronologically, over any extended period of time. In light of the dependency of the adrenals upon other tissues, such as the liver (3, 7), for their supply of ascorbic acid, it was deemed of interest to investigate the possible interrelationships between certain tissues by periodically assaying them for their vitamin C contents over a period of a few weeks following initiation of the deficiency diet feeding. Furthermore, although the use of paired-feeding technique serves to distinguish between effects wrought by reduced food intake and those directly resulting from the avitaminosis, little evidence has been presented for exclusion of adrenal ascorbate depletion due to non-specific stress other than inanition. Other indices of response to stress by the

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adrenal-hypophyseal axis, such as eosinopenia (11), might serve to make such a distinction.

This report describes the specific effects of a thiamine deficiency on the ascorbic acid contents of the adrenals, liver, and kidneys.

Experimental

The animals used in this experiment were 75-day old Holtzman rats with an initial weight of approximately 200 grams. After a short period of acclimatization, the animals were paired on a weight basis and separated into two groups. One group comprised the experimental animals which were fed, *al libitum*, a diet deficient in thiamine. The other group, the controls, received a complete diet, but only in amounts equal to that consumed by their experimental counterparts. A third unpaired group of four animals comprised starvation controls which were given water but no food. All animals were housed in individual cages with a wide mesh screen floor in order to prevent coprophagy. Analyses of the tissue levels of ascorbic acid were made on six different animals every other day for the duration of the twenty day experimental period. The animals to be analyzed were anesthetized with ether and weighed. A generous semi-circular incision was made in the abdominal wall to expose the underlying viscera. The adrenals were rapidly removed, followed by removal of one lobe of the liver and both kidneys. The excised organs were carefully trimmed of adhering adipose or other connective tissue and weighed to the nearest milligram on an analytical balance. Each of the organs was ground in a motor-driven tissue homogenizer for ascorbic acid extraction in cold 5% metaphosphoric acid. The ascorbic acid in the filtrate of the extracting medium was then determined by a modification of the method of Roe *et al.* (6).

Eosinophil counts were made daily for the first twelve days of the experiment by drawing samples of blood from the tail. These samples were then properly diluted and stained with an eosin-acetone solution and counted in a hemocytometer.

Results and Discussion

Within the first week of the experimental period the ascorbic acid concentration in the liver and kidneys of the experimental

animals dropped appreciably. The adrenals suffered a comparatively smaller decrease in ascorbic acid. In all experimental animals the hepatic ascorbate was less than that found in the controls, although the degree of difference gradually diminished toward the end of the experiment. Essentially the same pattern of depletion with diminution of the differences were seen in the renal values.

Figure 1 shows the relative decreases in ascorbic acid expressed as per cent change in the experimental tissues compared with the control levels. It can be seen that the large initial drop and subsequent changes in hepatic and renal ascorbate show considerable coincidence. The changes found in the adrenals are shown to be similar but of a smaller magnitude.

Inasmuch as little deviation from the concentration of adrenal ascorbic acid typically found in the normal rat was seen in the pair-fed controls, it would seem that the small decrease in food intake had little or no effect as a non-specific stress. Thus the depletion of ascorbic acid in the adrenals of the deficient animals may be assumed to be due to the thiamine deficiency *per se*. How-

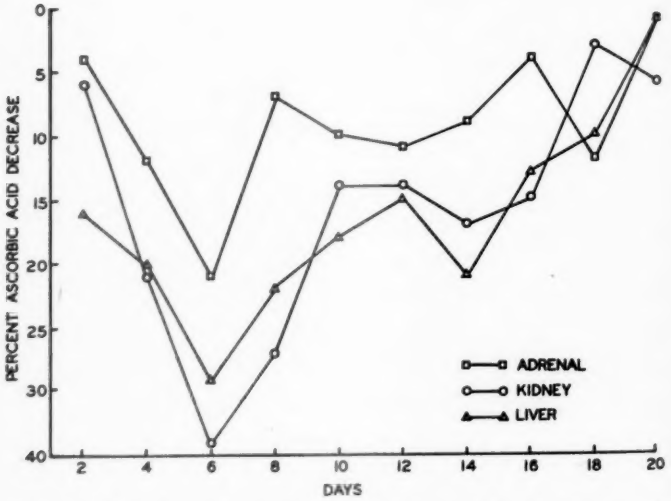

FIG. 1. The depletion of ascorbic acid in the tissues of the deficient animals expressed as per cent decrease by comparison with that found in their pair-fed controls.

ever, in order to make such an assumption it is necessary to rule out the possibility of adrenal depletion due to some other stress. If a decrease in the number of circulating eosinophils can be taken as an index of adrenal response to stress (11), the results indicate that such stress is not present, since no eosinopenia was found in the deficiency animals. On the contrary, the eosinophil counts were found to be higher in the deficiency animals than in their pair-fed controls. A marked eosinopenia was found in the starvation control animals by the second day. The mean eosinophil counts for the deficiency animals, the pair-fed controls, and the starvation controls were 234, 181, and 46 per cubic millimeter, respectively.

Inasmuch as the liver appears to be the main organ of synthesis of ascorbic acid in those mammals capable of synthesizing the vitamin, and since thiamine is known to affect this synthesis, at least in murid rodents, the initial drop in hepatic ascorbate was to be expected. Although the surprising concomitant drop in renal ascorbate was not anticipated, it can now be visualized that the kidney, and to a lesser extent, the adrenals, being unable to synthesize their own ascorbic acid, are directly dependent upon the liver to supply it. Thus a decrease in the supply results in a decreased tissue concentration of the vitamin. The close correspondence of the several curves, especially those of the liver and the kidneys, would seem to bear this out (Fig. 1).

The smaller extent of the vitamin C diminution in the adrenals compared with that of the liver and kidneys may be a manifestation of a superior ability of the adrenal to accumulate ascorbic acid in much the same way as leukocytes take up the vitamin despite a diminished plasma supply (1).

However, the effect of thiamine on the synthesis within the first week of the deficiency is not a lasting one since the tissue levels of vitamin C return to nearly normal by the twentieth day. It might be that the liver possesses an adaptive or compensatory mechanism for surmounting the defect by which the liver is initially affected in early thiamine deficiency.

Summary

Thiamine was shown to exert some influence on the ability of the liver to synthesize and supply ascorbic acid to other tissues such as the kidneys and the adrenals. It would not appear to be

essential in this respect, as such synthesis does not cease in the absence of dietary thiamine, and, in fact, returns to nearly normal by the end of twenty days.

There is considerable similarity in the fluctuations of hepatic and renal ascorbate in the experimental animals relative to the amounts found in their pair-fed controls. Similar, though more moderate, fluctuations are seen in adrenal ascorbic acid concentrations. Such changes probably reflect the dependence of these tissues on the liver for their supply of this vitamin.

The depletion of ascorbic acid in the adrenals of the deficiency animals within the first twelve days of the experiment is not thought to represent a response by the adrenal-hypophyseal axis to stress, as there was no coinciding eosinopenia found in the circulating levels of leukocytes in these animals.

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PREPARATION OF KIDNEY CELL CULTURES[a]

CRAIG WALLIS, RAYMOND T. LEWIS, AND JOSEPH L. MELNICK[b]

Monkey kidney cultures are widely used for the growth of human viruses, particularly those of the enterovirus group. It is important that the highest possible yield of kidney cells be obtained from these animals, which are difficult and costly to obtain. This report describes two modifications in procedure which have consistently produced 50% greater yields of cells and have allowed us to complete the trypsinizing process within a period of 60–80 minutes. The first modification is the use of the entire kidney without removing the pelvis; the second is the preparation and use of commercial trypsin in a more active form.

1. Use of whole kidneys. Removal of the pelvis of the kidney prior to trypsinization, as described by Youngner (1), has become standard in the procedure for dispersion and cultivation of monkey kidney cells (2). However, in stripping the pelvis a significant amount of tissue other than fibrous material is discarded. The use of the whole kidney not only gives larger yields of cells, but also reduces excessive exposure to environmental contaminants encountered during the stripping procedure.

During a period of 2 months, 60 young rhesus monkeys, each weighing about 5 pounds, were used for comparing yields from whole versus stripped kidneys. The average weight of both intact kidneys was 9.0 gm, and that of the stripped kidneys, 7.5 gm. The yield from the stripped kidneys of one monkey was similar to that obtained the past two years, averaging 1550 ml of suspension containing 300,000 cells per ml (see Table 1). However, the yield from the whole kidneys was 2100 to 2700 ml per monkey, with an average of 2350 ml, a 50% greater yield than that obtained from stripped kidneys. The increased yield obtained by processing whole kidneys seems to be due not only to the cells present in the tissue usually discarded but also to the swirling action created by the presence of the fibrous tissue. Cells are dispersed more rapidly by trypsin under these conditions, as com-

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TABLE 1

Comparison of Cell Yields Obtained from Whole Kidneys and Kidneys with Pelvis Removed

	Total Number of Monkeys Used	Average Yield as Volume of Cell Suspension per Pair of Kidneys	Average Yield as Number of Cells per Pair of Kidneys
Stripped Kidneys[a]	30	1550 ml*	4.65×10^8
Whole Kidneys[a]	30	2350 ml*	7.05×10^8

a Stripped kidneys averaged 7.5 grams per pair.
Whole kidneys averaged 9.0 grams per pair.
* 300,000 cells per ml.

pared to the tissue processed free of this fibrous material. In regard to cell growth and viral susceptibility, the quality of the cells obtained by trypsinizing the entire kidney was equal to that from stripped kidneys.

Recommended procedure: Kidneys are obtained aseptically from exsanguinated rhesus monkeys and the capsule removed. The entire kidney is placed in a tube and minced with uterine scissors into pieces about 5–7 mm in size. The material is then transferred to a trypsinizing flask, processed, and grown in M-H lactalbumin medium as described elsewhere (2), except for the modifications discussed below.

II. Preparation of trypsin solution. Trypsin purchased from commercial sources for the purpose of dispersing monkey kidney cells contains large quantities of bacteria and significant amounts of relatively insoluble material. Numerous freshly opened lots of trypsin from two sources (Difco and Nutritional Biochemical) were titrated by serial dilution for bacterial counts in tryptose phosphate broth tubes. Seven of eight lots tested contained between 5 and 50 million organisms per gram of dry trypsin. In the usual preparation of 0.2% trypsin, a turbid suspension is first obtained, which requires 2–3 hours on a magnetic stirrer before it approaches solution. Even after constant agitation, the suspension must be clarified by paper or other filtration procedure before any large amounts can be passed through a bacterial filter. During the 3–4 hours required for the preparation and filtration, the material may lose a portion of its enzymatic activity, and bacterial cytotoxins may be liberated into the solution. In addi-

tion, the filtration of large quantities of such a crude and contaminated suspension poses difficulties in regularly obtaining sterile filtrates with ordinary laboratory equipment.

The enzyme trypsin itself is very soluble, dissolving within 10 minutes even in concentrations as high as 10%. The insoluble and slowly soluble material present in commercial preparations of *1:250* and *1:300 trypsin* can be eliminated by centrifugation, thereby also excluding from the stock an excess of bacteria and other undesirable organic constitutents which have negligible trypsin activity.

Recommended procedure: A 10% suspension of trypsin powder is made in a cold buffer of salts and glucose containing the following ingredients (as grams per liter): NaCl, 8.0; KCl, 0.4; Na_2HPO_4, 0.06; KH_2PO_4, 0.06; and glucose, 50.0. The suspension is stirred on a magnetic unit for 10 minutes at 4° C. It is then centrifuged at 10,000 rpm for 1 hour at 4° C, yielding a clear, brownish solution as the supernate. This represents a 50× concentrate which is then filtered through a Seitz S-3 filter (144 mm) within 30 minutes. It is dispensed aseptically and stored in the frozen state until ready for use.

The trypsin diluent consists of the same salts at the same concentrations as described above, except that glucose is omitted. This diluent is sterilized by autoclaving, and upon cooling $NaHCO_3$ is added to make a 0.05% solution. Just before use the concentrate is thawed and diluted 50-fold with the diluent, to make a working solution of 0.2% trypsin. Starting with 100 grams of commercial trypsin, this procedure yields 50 liters of 0.2% trypsin as needed, with elimination of laborious filtration procedures.

Numerous quantitative tests have been performed on the trypsin solution to determine its proteolytic activity against 5% milk (Pet). No detectable activity was lost by subjecting the 10% solution to the sedimentation and filtration procedure outlined. Furthermore, the insoluble sediment, consisting of about 50% by volume of the starting material, contained low or negligible proteolytic activity (see Table 2).

The concentrate is maintained at pH 5.9 by the buffer. After storage for at least 3 months, it consistently has produced working solutions of high potency. The cultures obtained with such preparations are of equal quality to those processed by trypsin

TABLE 2

Retention of trypsin activity in the highly soluble fraction

Processing of Trypsin (All procedures carried out at 4° C.)	Titer*
0.2% suspension stirred for 1 hour	16,000
10% suspension stirred for 1 hour	16,000
10% suspension stirred for 10 minutes, centrifuged at 10,000 rpm for 1 hour	
A. Supernate	16,000
B. Sediment (50% by volume), resuspended and stirred for 1 hour	< 100

* Based on concentration of original trypsin powder. Serial dilution of trypsin made in cold phosphate-buffered saline, and equal volume of 5% Pet milk added to all tubes. Incubated at 37° C. for 1 hour. End point is last tube clarified.

which had been filtered in the form of the 0.2% working solution (1,2).

Summary

Two modifications of the trypsinization procedure (1, 2) for preparing primary suspensions of kidney tissue have been introduced. By using the entire kidney without removing the pelvis, and by using a more easily prepared and more active trypsin solution, final cell suspensions have been obtained in 60 to 80 minutes, and the cell yield has been increased 50 per cent.

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CYTOCHEMICAL STUDIES ON PROTEINS IN THE RETINA[a]

M. YOSHIDA[b]

According to Wald and Brown (1952), opsin, the protein component of the visual pigments, is bound to sulfhydryl groups which are liberated when rhodopsin is bleached. The outer segments of rods and cones in various vertebrate retinae are acidophilic and show a strong reaction for proteinbound sulfhydryl groups (Wislocki and Sidman, 1954; Sidman and Wislocki, 1954). In spite of the predominance of protein-bound sulfhydryl groups in the outer segments revealed by histochemical methods, it is not clear whether they are exclusively associated with opsin.

This study was designed to detect the chemical nature of proteins localized in the outer segments of visual cells in some vertebrate retinae.

Materials and Methods

Freshly excised retinae of oxen and frogs, *Rana catesbiana* and *Polypedates buergeri*, and of the fresh-water fish, *Odontobutis obscura*, were used for this study. They were fixed with Carnoy's fixative for one hour. Retinal sections from the four species were attached to the same slide with egg albumin for comparison of the stainability of proteins. Histone was removed from retinal sections by incubation in a pepsin solution (3 mg/ml in 0.02 N hydrochloric acid) for 90 min. at 37° C. (Kaufmann et al., 1951). Nucleic acids were extracted from sections with hot 5 per cent trichloroacetic acid (TCA) for 15 min. The Millon test based on the method of Pollister and Ris (1947) and the mercuric bromphenol blue (BPB) method (Mazia et al., 1953) were applied to demonstrate the localization of proteins in retinal sections treated with 5 per cent TCA or pepsin, and in control sections.

Extinction measurements of non-histone protein stained by the sulphuric acid-Millon reaction were made at 480 mμ with a microspectrophotometer. The absorption spectrum of the dye (BPB)-protein complex was measured in the visible light range from 500 to 750 mμ at ten mμ intervals. The method for microspectrophotometric measurement was previously reported (Yoshida, 1958).

[a] A part of this work was reported at the Thirty-fifth Annual Meeting of the Physiological Society of Japan (May, 1958).

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1. *Non-histone protein in the outer segments.*

The extinction of non-histone protein stained by the sulphuric acid-Millon reaction was measured with the aim to detect differences in the structural compositions and quantities of proteins localized in the outer segments of rods and in the nuclei of the visual cells. Moderate staining of non-histone protein was observed in all parts of the retina of *Rana catesbiana*. Retinal elements, such as the inner and outer plexiform layers, nuclei distributed in three nuclear layers, and Müller's fibers were not clearly distinguishable due to their weak stainability. On the other hand, the color tint was relatively intense in the regions of the outer segments of the rods. The mean extinction of non-histone protein was 0.125 in the outer segments and 0.092 in the nuclei. These extinction values represent averages of 30 measurements.

2. *Localization of proteins stained with the mercuric BPB reagent.*

In a preliminary experiment, tissue sections from various organs of *Megalobatrachus japonicus* were stained with BPB (with or without mercury) in order to test the stainability of proteins. In the kidney, for example, both cytoplasmic and nuclear structures of cells in the regions of the uriniferous tubule were stained moderately, except for the nuclei of erythrocytes which showed strong and homogeneous blue tints. However, as is indicated in Fig. 1, the nuclear structure was distinctly identifiable with an increased color intensity, when the sections were treated with 5 per cent TCA and stained with the mercuric BPB reagent. The stainability with mercuric BPB thus observed was in agreement with data reported by Mazia *et al.* (1953).

In the retina, the mercuric BPB reaction gave a greater optical density and sharper morphological definition than the Millon reaction. Maximum extinction measured at the outer plexiform layer which showed a rather intense tint was at approximately 610 mμ, as is illustrated in Fig. 2. The curve obtained was unimodal and fairly symmetrical. The stainability with BPB between ox and frog retinae was compared in sections attached to the same slide and stained simultaneously. As is shown in Fig. 3, the outer segments of rods and cones in the ox retina were rather intensely stained as compared to the other elements of the retina,

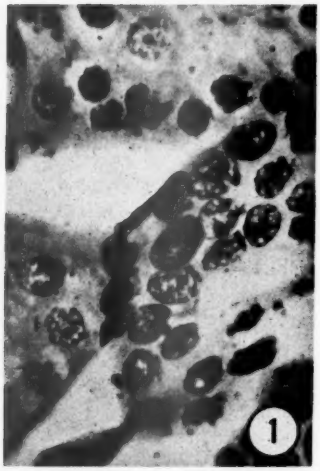


FIG. 1. Kidney of *Megalobatrachus japonicus*. Stained with the mercuric BPB reagent after removal of nucleic acids. X 500.

although a moderate staining was also observed in Müller's fibers. The strongest staining was obtained in the myoids of cones.

All retinal elements of frogs were clearly distinguishable in various color intensities after staining with BPB, as is indicated in Fig. 4. The inner and outer plexiform layers, external limiting membrane, inner and outer segments of rods and cones, and Müller's fibers were intensely stained, while the intermediate plates between the inner and outer segments of rods and cones were not stained at all. In untreated retinae the staining of all nuclei was weak and uniform so that no regional differences could be observed. However, after extraction of nucleic acids from the sections, the color intensities in all three nuclear layers were enhanced (Fig. 5). The color intensity of the nuclei in the inner nuclear layer was greater than the nuclei of the other layers. The color intensity of the outer segments of rods and cones was not impaired by the treatment with TCA. Similarly, the color intensity in the inner and outer segments of rods and cones of frog retinae was not impaired after removal of histone with pepsin (Fig. 6). On the other hand, the color tints in the rest of retinal elements, especially in the nuclei were found to be decreased after pepsin digestion. The external limiting membrane

showed a fairly sharp staining, even after pepsin digestion. In the retina of the fresh-water fish, *Odontobutis obscura*, the BPB reagent gave similar results as in those of frogs and oxen. The inner segments of cones as well as the filamentous outer segments of rods showed a fairly intense staining with the mercuric BPB reagent. The tendency for enhanced staining of the retinal nuclei after the removal of nucleic acids was found to be identical with that observed in frog's retinae (Fig. 7). After extracting histone from retinal sections with pepsin, the color intensity was observed to decrease in the nuclear layers without showing an impairment in the color intensity in the inner and outer segments of rods and cones (Fig. 8).

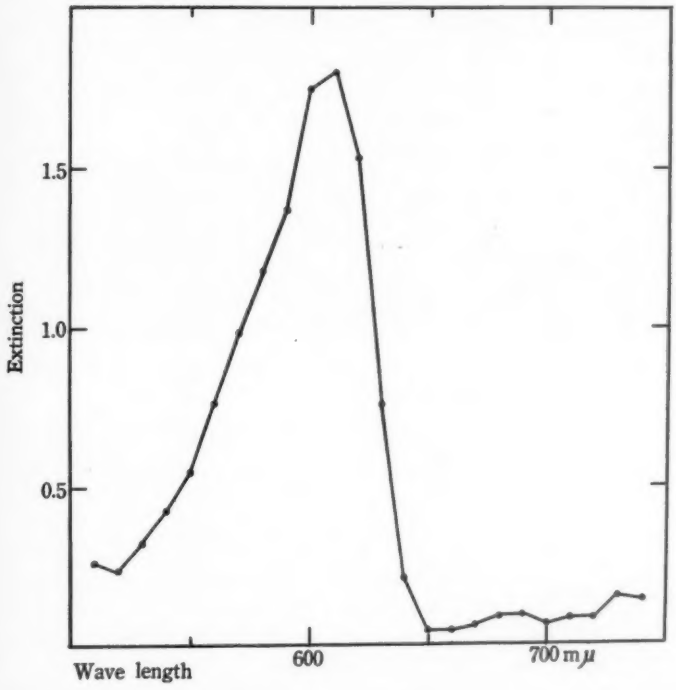
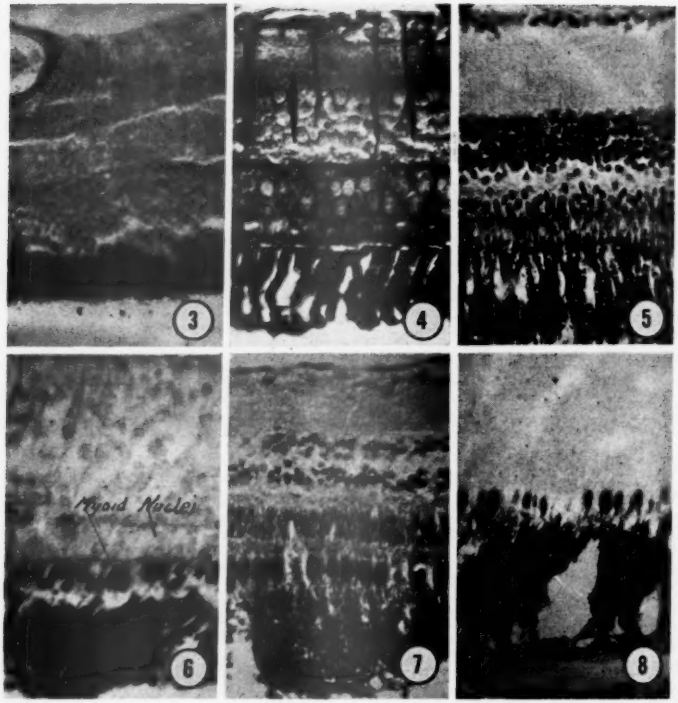


FIG. 2. Absorption curve of the mercuric BPB-proteins measured at the outer plexiform layer of the frog's retina, *Rana catesbiana*.



FIGS. 3–8. Photomicrographs showing the localization of proteins in the retinae, stained with the mercuric BPB reagent.

FIG. 3. Ox retina. Note the intense staining at the outer segments of rods and cones. X 150.

FIG. 4. Frog's retina, *Rana catesbiana.* Retinal elements with various intensities in staining are seen. Note the intense staining in the outer segments of rods and cones, and in Müller's fibers. X 320.

FIG. 5. Frog's retina, *Polypedates buergeli.* Pretreated with hot 5 per cent trichloroacetic acid. X 270.

FIG. 6. Frog's retina, *Rana catesbiana.* Pretreated with pepsin. Note the intense staining in the outer segments of rods and in the myoids of cones. X 335.

FIG. 7. Retina of a fresh-water fish, *Odontobutis obscura.* Pretreated with hot 5 per cent trichloroacetic acid. X 275.

FIG. 8. Retina of a fresh-water fish, *Odontobutis obscura.* Pretreated with pepsin. X 275.

Discussion

Serra (1946), using a modified Millon reaction, demonstrated the presence of tyrosine in the chromosomes and nucleoli of frog ovarian tissue. Arginine-rich protein was found to be present in the chromosomes and nucleoli of rabbit liver and of Ambystoma testis (Thomas, 1946). Pollister and Ris (1947) measured cytophotometrically the content of non-histone protein in the nuclei of pollen mother cells of *Zea mays*. Their findings were based on the fact that histone can be extracted with a solution of sulphuric acid and mercuric sulfate (Mirsky and Pollister 1946). In the present experiment, with the microspectrophotometer, the outer segments of rods were found to contain more non-histone protein than the nuclei of visual cells in frog retina. Distortions of the layer structure, due to fixation and dehydration shrinkage, may be responsible for the relatively great deviation from the mean during extinction measurements.

The sharp staining of proteins with the mercuric BPB reagent permitted a clear identification of the retinal elements. In the retina, the mercuric BPB reagent selectively stained cytoplasmic structures, especially the cytoplasm of Müller's cells and the rod outer segments. Nuclear structures were faintly recognizable. It is likely that proteins located in the outer segments of visual cells are mainly basic, because proteins in those segments were intensely stained even after omission of mercury, as reported by Mazia *et al.* (1953). My results seem to be in agreement with the data presented by Wislocki and Sidman (1954), who described acidophilic protein in the outer segments of visual cells. According to Mazia *et al.* (1953), other proteins also react with BPB in the presence of mercury. The results presented here confirm that the stainability or color intensity in the outer segments and myoids of visual cells is not influenced by mercury. Therefore, both basic and acidic proteins and peptides in the inner and outer segments are stainable with the mercuric BPB reagent; possibly there is no selective stainability with standard BPB staining method of proteins located in those segments. A similar color intensity was found in the rod outer segments before and after removing histone by pepsin digestion. No changes in the color tints in the outer segments were observed after nucleic acid extraction. Therefore, the fact that the constant staining in the

outer segments was maintained after removal of histone and nucleic acid indicates that the outer segments contain inconspicuous amounts of both substances.

The maximum extinction of the dye (BPB)-proteins was in agreement with data by Mazia *et al.* (1953), who stated that the preparation stained by this procedure followed the Beer-Lambert Law in microspectrophotometric measurements. However, quantitative measurements were not made in the present expriment, because inconstant stainability in the retinal structures due to the washing procedure made this impractical (compare Bonhag, 1955).

The enhanced staining was confined to the retinal nuclei after the removal of nucleic acids from sections. According to Mazia *et al.* (1953), the increased staining of chromosomes following the extraction of nucleic acids is consistent with the view that many of the basic groups of nuclear proteins are bound to the acid groups of nucleic acids. Ficq (1955) observed enhanced staining of ovarian tissue of *Rana fusca* with this dye after removing ribose nucleic acid by ribonuclease digestion.

It is of interest that the enhanced staining in the retinal nuclei gives a close resemblance to the color intensity of the nuclear staining with the Feulgen reaction; after the removal of nucleic acids, the color tints in the retinal nuclei were markedly altered, whereby the nuclei in the inner nuclear layer showed the greatest intensity of color. This may indicate that, after removal of nucleic acids, a constant relationship in the nuclear basic protein content exists in all retinal nuclei stained with the mercuric BPB reagent.

Summary

The localization of proteins in the retinae was demonstrated by applying the sulphuric acid-Millon and mercuric BPB reactions. The results obtained are as follows:

1. Non-histone protein was observed to be rich in the outer segments of rods in frog's retinae.

2. The sharp staining of proteins with the mercuric BPB reagent permitted a clear identification of the retinal elements. The color intensity with mercuric BPB was stronger in the inner and outer segments of rods and cones than in the rest of retinal elements. Staining reaction was more marked in the retinae of frogs in contrast to the retinae of oxen.

3. After extraction of nucleic acids from the retinal sections, enhanced color intensity was observed in the nuclei of retinal cells.

4. The color intensity of the retinal nuclei which stained with BPB after extraction of nucleic acids suggests a constant relationship in the contents of nuclear protein coupled with the mercuric BPB.

5. After removal of histone from the sections, the color intensity with BPB was unchanged in the outer segments of rods and cones, whereas the other retinal layers showed considerably lower color intensity.

6. No changes in the color intensity with BPB were observed in the outer segments of rods and cones following treatment with hot TCA.

Acknowledgments

The author wishes to express his sincere gratitude to Professor T. Kawamura, of the Zoological Laboratory, Hiroshima University, for valuable suggestions. He is also greatly indebted to Professor W. Hild of the Department of Anatomy, The University of Texas Medical Branch, for his assistance in the preparation of the manuscript.

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BOOK REVIEWS

General Cytology, Third Edition, by E. D. P. De Robertis, W. W. Nowinski, and Francisco A. Saez, W. B. Saunders Company, Philadelphia and London, 1960. xvi + 555 pages, $10.00.

Publication of this thoroughly revised third edition of a well-known textbook is an event of first magnitude for cell biologists, whether or not they are academically involved in transmitting enthusiasm for their subject to studious youth. The sure reception this book will meet throughout the world was seen in Paris, at the September, 1960 International Congress of the Society for Cell Biology. Within two hours of the time a first copy was put on display among other scientific books, it had been purloined, and author De Robertis found it necessary to erect a sign requesting self-restraint from the assembled cytologists.

The book begins with a history of cytology and an account of the philosophical considerations attending its early years. It proceeds through the chemical and physico-chemical organization of protoplasm and a description of the methods used to study cells to the cytochemical and submicroscopic organization of the cell. The several components of the cell are then discussed, from the ground cytoplasm, Golgi complex, mitochondria, and plasma membrane to the nucleus, and through growth and cell division the reader is taken from mitosis to meiosis and cytogenetics. After a chapter on enzymes and cell metabolism, more complex aspects of cellular activity are covered, and the final chapter is concerned with differentiation and senescence of the cell. In all, there are 17 chapters, as compared with 12 in the previous edition. The plant cell and chloroplasts are accorded more attention in this third edition. Unlike the earlier editions, in this one each chapter represents the combined efforts of all the authors, and the greater coherence and comprehensiveness are readily evident.

The book has grown with the years, is remarkably up-to-date, and is excellently illustrated, especially with a choice selection of electron micrographs from the rapidly maturing field of cellular fine structure. The authors are to be congratulated for a superb synthesis of a large and fast-growing field and the publishers for a handsomely well-made book.

John J. Biesele

Human Pituitary Hormones. Ciba Foundation Colloquia on Endrocrinology, Vol. XIII. Ed. G. E. W. Wolstenholme and C. M. O'Conner. Little, Brown and Company, Boston. $9.50. 321 pp.

This volume includes the papers presented by 15 participants at the colloquim held in Buenos Aires on August 6–8, 1959. It brings together important contributions to our knowledge of the hormones of the anterior pituitary gland.

Recent advances in the chemistry and immunology of growth hormone obtained from several species including man are reviewed. All told, about one-half of the volume deals with the important recent studies made on growth hormone. Several reports of the clinical and metabolic effects obtained with human growth hormone preparations should be of interest to clinicians. Chapters dealing with the elucidation of the structure of ACTH and MSH from human pituitary glands signal the rapid advances now being made by the chemists working in this field. The lively discussions which followed the presentations are well worth reading. This colloqium was held in honor of Professor B. A. Houssay and its published record is a volume that will be of wide interest to all students of Endocrinology.

Edward G. Rennels

Coprologie Parasitaire Humaine, J. Bailenger. Imprimerie: E. Drouillard, Bordeaux. 1958, pp. 277; 173 figs.

The current need for a broad survey of coprological methods in human parasitology will be reasonably well satisfied by this book. Although appropriate as a supplementary reference text for students in introductory parasitology courses, it will be most valuable for those preparing for advanced examination in the field. The French is clear and direct and should not deter the student with only a limited knowledge of this language. The principal defect is that citations are to author and year with no literature references provided. Methods of preparing helminths for examination are briefly reviewed, keys are given to the common species as well as descriptions of the important characteristics. Numerous photographs and line drawings are included, the former of only fair quality reproduction and the latter diagrammatic, but both are adequate for comparative purposes. Methods for examining stools for helminth eggs are reviewed in considerable detail with pertinent comment on the advantages and disadvantages of each for various purposes. A key to nematode larvae includes the main points of differentiation, but there are some deficiencies. For example, an inexperienced observer would find the comparison of the rhabditoid larvae of the hookworms and *Strongyloides* inadequate.

Photographs of eggs, cysts and trophozoites as well as other objects which puzzle the beginning microscopist are numerous and adequate. Examination methods for protozoa are given in detail with emphasis on staining procedures. Keys to cysts and trophozoites are accompanied by numerous photographs. In some difficult species the photographs are only adequate for review purposes after the student has compared living material with better reproductions. Fungi, arthropods and crystals commonly encountered are described and figured. Food residues and means of identifying them are well covered. A number of protozoological culture methods are given but the coverage is not exhaustive. Similar methods for fungi seem more adequate. Egg counting methods for helminths include only the original method of Stoll and that of Caldwell, with none of the more recent methods or the modifications of the former method. A few methods for chemical analysis are described, as well as a brief mention of rectal biopsy for schistosomiasis.

The book ends with an extensive alphabetical dictionary including names of parasites, artifacts, and other substances found in feces with brief summaries or page references to longer descriptions in the text. There is combined index of authors and subjects. A unique feature is the attachment by a cord of a bookmark with photographically reproduced scales by which the drawings and photographs at higher magnification can be measured. In spite of the inadequacies mentioned, this book would be a valuable addition to the parasitologist's reference shelf.

J. A. Scott

Congenital Malformations. Ciba Foundation Symposium. G. E. W. Wolstenholme and C. O'Connor, 308 pps. Little, Brown and Co., Boston, Mass. $9.00.

This is the twentieth in the series of General Symposia. The twenty-nine participants were selected from widely disparate disciplines and widely separated institutions. Despite this the symposium is only a partial coverage of materials upon which they have been working. It is not a review of the subject and much important material is omitted.

The attempt to define congenital anomalies for purposes of classification is difficult since anomalies range from gross to microscopic, from biochemical patterns to antibody reactions. It becomes clear that many of them are related to gross chromosomal losses or gains, translocations and nondisjunctions. But many genetic mutations come without visible chromosomal alteration. No one yet knows the path from gene to metabolic enzyme.

The experimental production of anomalies offers more possibilities. It is granted that timing is important but there seems to be a general lack of knowledge even among the participants as to the physiological history of embryonic primordia which determines this time specificity. The effects in general are arrests, local or general with some attempt at regulation after the incident force is removed. These agents may act as antimetabolites or blocks of either oxidative or synthetic processes. Outside of timing there is little specificity of result and what there is is not understood.

The study of the etiology of stunted development producing runts in otherwise normal litters is a valuable approach involving the serial timing of implantations and the normality of the vascular supply. Hydramnios also raises interesting questions as to origins and content of amniotic fluid.

The participants agreed that we are still a long way from an analytical approach to the problems of teratology. The mechanism by which one of two products of mitosis takes a separate line of differentiation from its mate, even in tissue culture, is still unknown. If chromosome DNA constitution has any part to play in serial differentiations all experimental alterations will of necessity be referred to it. It does not seem probable.

John G. Sinclair

Man and His Body. Benjamin F. Miller and Ruth Goode. 376 pp. Simon and Schuster, New York, $5.95.

This book attempts to produce an ontogenetic and physiological picture of the human body for the intelligent layman. The chapters are named with

attractive titles and arranged in logical sequence from a beginning in embryology, through biochemistry and cytology to a finish in neuroanatomy and psychology. There are a few semi-diagrammatic drawings so that comprehension will depend upon the text. The text is far more physiological than anatomical.

The usual conventional scientific description is modified into interesting literary constructions by use of analogies and the elimination of unnecessary facts. Significant materials are retained. A voluminous index makes its use as a reference book quite possible. While there will be some quibble by specialists over acceptance or interpretation of some facts, the text is astonishingly complete and correct. Specialists reading and commenting on the several chapters undoubtedly helped but the unity of presentation achieved is a compliment to the authors and a great help to readers.

John G. Sinclair

Survey of research in gestation and the developmental sciences. Jack Davies. 203 pp. Williams and Wilkins Co., Baltimore.

The title "survey" is more correct than review. In the author's introduction he states that at the request of the chief of the Division of the Medical Sciences, Naional Institutes of Health a study was made of the reports coming in from the many grants made in this field for some years. The interests included cover most of the field of development particularly in mammals. However a good deal of work carried out without the aid of grants in the earlier years of the period covered, is missing.

The striking advance during the later years has been the invention and application of analytical techniques, often adapted from physics and chemistry. The bibliographies of these techniques are quite complete and are a valuable compilation for investigators. Most of the general reviews are also mentioned in references.

A listing of titles gives an idea of coverage: Germ cell and fertilization, Implantation, Placenta, Fetal fluids, Embryo and fetus, Teratology, Immunology and pregnancy, Parturition and lactation, special problems of gestation. The present status of open problems is well stated.

Publication costs were apparently absorbed by the Association for the Aid of Crippled Children. Price?

John G. Sinclair

CALLING ATTENTION TO

OCTOBER, 1960

Professional Relationships: Well-written & important is RH Blum's *The Management of the Doctor-Patient Relationship* (McGraw-Hill, New York, 1960, 304 pp, $8.5). Useful also is WF Bowers' *Interpersonal Relationships in the Hospital* (CC Thomas, Springfield, Ill, 1960, 132 pp, $5). Then there's OF Ehrentheil & WE Marchand discussion on *Clinical Medicine & the Psychotic Patient* (CC Thomas, Springfield, Ill, 1960, 405 pp, $10.75). Interesting is LW Frohlich's comments on USA physicians & the pharmaceutical industry (*Proc Roy Soc Med* 53/579/60).

Primatologia: Under editorial direction of H Hofer, AH Schultz & D Starck, the *Handbook of Primatology* began to appear in 1956 (Vol 1, *Systematics, Phylogeny & Ontology*, with Vol III part 1 on the alimentary tract & urogenital organs in 1958). Now comes Vol III part 2 on blood & circulatory organs, respiratory organs and teeth (S Karger, Basel, 1960, 880pp, 371 illus, $48), & Vol II part 5 on the ear (*ibid*, 78 pp, $4.3).

Preclinical: EB Babskii & Co discuss interrelationship of cardiac minute volume & peripheral resistance in humans (*Doklady: Biol Sect* 130/143/60). GK Davis edits symposium on interaction of mineral elements in nutrition & metabolism (*Fed Proc* 19/635–679/60). H Davis chairs symposium on neuropharmacology (*ibid* 611–634). BM Gekht & Co note variations in serum copper in diencephalic lesions (*Doklady: Biol Sect* 130/149/60). T Hayashi edits symposium on *Subcellular Particles* (Ronald Press, New York, 1960, 213pp, $6). HJ Hurley & WB Shelley consider *The Human Apocrine Sweat Gland in Health & Disease* (CC Thomas, Springfield, Ill, 1960, 138pp, $6.5). AB Lerner & JD Case find melatonin is hormone of pineal gland (*Fed Proc* 19/590/60). D Nachmansohn edits important symposium on *Molecular Biology: Elementary Processes of Nerve Conduction & Muscle Contraction* (Academic Press, New York, 1960, 177pp, $6.5). EF Polazhaev correlates EEG changes with development of cortical co-ordination (*Sechenov Physiol J USSR* 46/31/60). D Rudnick edits symposium on *Developmental Cytology* (Ronald Press, New York, 1960, 213pp, $7). A Szent-Gyorgyi gives basic theory in an *Introduction to a Submolecular Biology*, with stimulating ideas as usual (Academic Press, New York, 1960, 135pp, $5). V Wartiovaara & R Collander discuss *Permeabilitatstheorien* (Springer, Wien, 1960, 100pp, DM 29).

Clinical: J Adriana reviews clinical pharmacology of local anesthetics (*Clin Pharmacol Therap* 1/644/60). EA Carr edits useful symposium on clinical use of antimetabolites (*ibid* pp 554–589). BL Coley revises 2nd edition of *Neoplasms of Bone & Related Conditions* (Hoeber, New York, 1960, 878pp, $30). RR Ellison & JH Burchenal discuss treatment of chromic granulocytic leukemia with 6-substituted purines (*Clin Pharmacol Therap* 1/629/60). A Hollaender edits symposium on radiation protection & recovery (*Fed Proc* 19/562–589/60). JW Hurst edits Emory University symposium on *Cardiac*

Resuscitation (CC Thomas, Springfield, Ill, 1960, 150pp, $5.5). LS James reviews effect of pain relief for labor & delivery on fetus & new-born (*Anesth* 21/405–430/60, with 144 references). SD Larks describes *Electrohysterography: the Electrical Activity of the Human Uterus in Pregnancy & Labor* (CC Thomas, Springfield, Ill, 1960, 136pp, $5.75). HJ Moersch & HA Anderson discuss *Diagnosis & Treatment of Diseases of the Trachea & Bronchi* (CC Thomas, Springfield, Ill, 1960, 115pp, $4.25). HR Viets & RS Schwab review *Thymectomy for Myasthenia Gravis* (CC Thomas, Springfield, Ill, 1960, 158pp, $7). WG Walters considers therapeutic applications of conditioning theories in psychiatry (*Proc Roy Soc Med* 53/495/60).

Of Real Interest: LD Adams gives much & well in *The Commonsense Book of Wine* D McKay Co, New York, 1960, 192pp, $3.5). NK Covalt discusses well the meaning of religion to older people (*Geriatrics* 15/658/60). J Duffy neatly describes the tough New Orleans career of Erasmus Darwin Fenner (1807–1866) medical journalist & educator (*J Med Educ* 35/819/60). DJ Ingle has a ball, & it's a honey, on Percy T Diorets, Endocrinologist—a Fable, in which many legs are pulled, & who else appreciates the poem on friendship by Craik (sic), or the unpublished ones? (*Endocrinology* 67/139/60,—& how did this get past the ed?). Grim & unpleasant but provocative is H Kahn's Rand Corporation report *On Thermonuclear War* (Princeton Univ Press, Princeton, N.J., 1960, 288pp, $10). FSC Northrop is provocatively rambling in *Philosophical Anthropology & Practical Politics* (Macmillan, New York, 1960, 394pp, $6.5). FNL Poynter offers a pleasant account of the relations of John Donne & William Harvey (*J Hist Med* 15/233/60).

NOVEMBER, 1960

Clinical: AD Bass & GK Moe edit symposium on *Congenital Heart Disease* (AAAS, Washington, 5, D.C, 1960, 363pp, $5). G Bergmann & Co edit huge 9th vol in 6 parts of *Hndbk Inner Med* on *Herz & Kreislauf* (Lange & Springer Berlin, 1960, 750pp, 1710 illus, DM 1096). SM Bluefarb doubles in the *Cutaneous Manifestations of Benign Inflammatory Reticuloses*, and in *Leukemia Cutis* (CC Thomas, Springfield, Ill, 1960, 352pp, 114 illus, $10 & 536pp, 170 illus, $18.5 respectively). Useful reference source is RH Durham's *Encyclopedia of Medical Syndromes* (Hoeber, New York 16, 1960, 434pp, $13.5). JS Hanrahan & D Bushnell analyse *Space Biology: The Human Factors in Space Flight* (Basic Books, New York, 1960, 263pp, $6). Sound, scientific & not-to-be-snooted-at is MJ Hirsch & RE Wick's well edited *Vision of the Aging Patient: An Optometric Symposium* (Chilton, Philadelphia, 1960, 343pp, $7.5). JP High & Co find Fluphenazine a potent & relatively safe tranquilizer (*Toxicol Appl Pharmacol* 2/540/60). LE Hollister notes sense & nonsense in "placebology" (*Current Therap Res* 2/477/60). ED Pellegrino & SJ Farber note changes in mineral composition of bone in various clinical disorders (*J Lab Clin Med* 56/520/60). J Schmid well covers *Neuraltherapie* (Lange & Springer, Berlin-Wilmersdorf, 1960, 290pp, DM 42). LD Vandam & FD Moore review adrenocortical mechanisms related to anesthesia (*Anesth* 21/531/60). GEW Wolstenholme & C O'Connor edit Ciba

Symposium on Congenital Malformations (Little Brown, Boston, 1960, 308pp, $9).

General: AR Dave notes intriguing aspects of hibernation (*Sat Rev*, Oct 1/60, p48). LD Davis makes sense in *The Commonsense Book of Drinking* (David McKay, NY, 60, 210pp). ND Fabricant describes *13 Famous Patients*, all recent (Chilton, Phila, 60, 231pp, $4). RK Goldsen & Co find *What College Students Think* (Van Nostrand, Princeton, NJ, 1960, 322pp, $6). J Kobler sympathizes with *The Reluctant Surgeon: A Biography of John Hunter* (1728–1793), *Medical Genius* (Doubleday, Garden City, NY, 60, 359pp, $4). WJ McGlothlin gives *Patterns of Professional Education* (Putnam, NY, 60, 288pp, $7). W Penfield novelizes Hippocrates in 5th Cent BC Cos, in *The Torch*, with scholarly notes (Little Brown, Boston, 60, 364pp, $4). ER Pinckney says *You Can Prevent Illness* (Lippincott, Phila, 60, 157pp, $3.5). MK Sanders edits sharp scathing supplement on *The Crisis in American Medicine* (Harper's Oct 1960, pp 121–168).

Basic: W Arnold & RK Clayton give evidence for electronic factors in photosynthesis (*Proc Nat Acad Sci* 46/769/60). NK Barkov notes analgesic properties of phenothiazine derivatives (*Farmakol Toksikol* 23/307/60). JM Coon & EA Maynard edit symposium on problems in toxicology, covering air 7 water pollution, food additives, radiactivity, space hazards (*Fed Proc* 19; No 3, pt II, Suppl 4, 52pp). H Hermann discusses direct metabolic reactions between animal cells (*Science* 132/529/Aug 28/60). PD Johnson studies autoregulation of intestinal blood flow (*Amer J Physiol* 199/311/60). J Mead analyses control of respiratory frequency (*J Appli Physiol* 15/325/60). KF Nakken & Co study inactivation of penicillin by cysteine & other mercaptamines (*Biochem Pharmac* 3/89/60). D Quivy reviews humoral & nervous regulatory mechanisms of blood coagulation (*J Phyiol Paris*, 52/607/60). H Reichel well surveys *Muskelphysiologie* (Lange & Springer, Berlin-Wilmersdorf, 60, 288pp, DM 58). M Reichlin & WJ Harrington demonstrate erythropoietin in normal plasma (*Blood* 16/1298/60). IH Scheinberg & I Sternlieb analyse copper metabolism (*Pharmacol Rev* 12/355/60). RF Sognnaes edits *Calcification in Biological Systems* (AAAS, Washington 5, DC, 60, 525pp, $8). E Sutherland & TW Rall show relation of adenosine phosphate & phosphorylase to catecholamine action (*Pharmacol Rev* 12/265/60). J & HM Tepperman well review effects of hormones on cells with note on pinocytosis (*Pharmacol Rev* 12/301/60). NA Vershbinskaya notes inheritable factors in adaptation of animal tissues to anoxia (*Doklady: Biol Sect* 130/159/60). PG Waser offers much on cholinergic receptors (*J Pharm Pharmacol* 12/577/60). GEW Wolstenholme & CM O'Connor edit important Ciba Symposium on *Human Pituitary Hormones* in honor of BA Houssay (Little Brown, Boston, 1960, 336pp, $9.5).

Psychiatry et al.: HA Abramson edits conference on *The Use of LSD in Psychotherapy* (Macy Foundation, New York, 60, 304pp, $4.5). FR Donner & FW Schueler study amnesic properties of scopolamine (*Arch Internat Pharmaco Therap* 127/449/60). F Flugel edits symposium on neuropsychopharmacology (*Med Experi* 2/58/60). RJ Plunkett & JE Gordon review

Epidemiology & Mental Health (Basic Books, New York. 60, 126pp, $2.75). H Selbach & Co chair symposium on monoamine liberators & monoamine-oxidase inhibitors (*Psychia Neurol* 140/1–253/60). D Svorad & V Novikova note experimental insomnia does not affect neurotic state (*Sechenov Physiol J USSR* 46/67/60). E Weigert considers loneliness & trust as basic factors of human existence (*Psychiatry* 23/121/60). J Wortis edits *Recent Advances in Biological Psychiatry* (Grune & Stratton, New York, 60, 431pp, $10).

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